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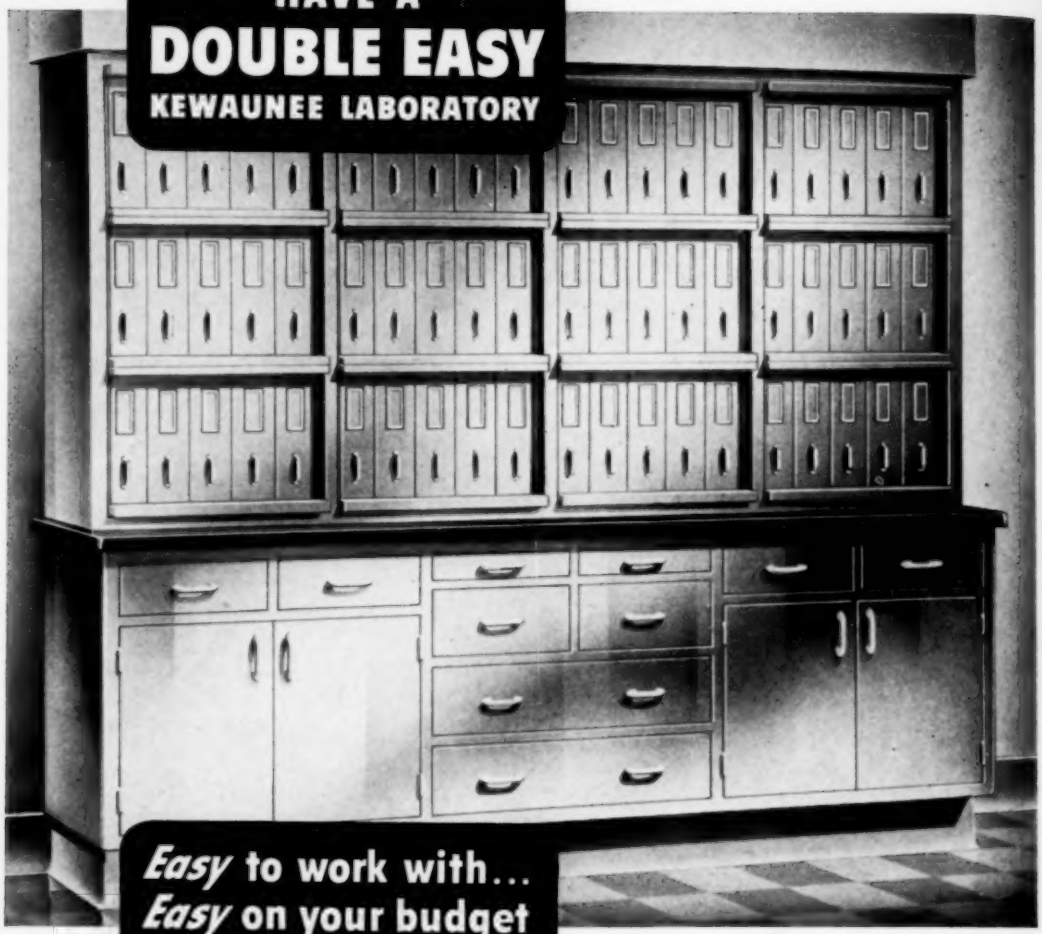

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VOLUME XXXVII — *JUNE, 1948* — NUMBER 6 — CONSECUTIVE No. 12

Assay of Heparin. II. Influence of Various Factors on Potency and Clotting Concentration of Heparin*,†,‡

By R. H. K. FOSTER

In assaying commercial samples of heparin no difference in potency was found when tested on either fresh or frozen beef or sheep plasma nor was any seasonal influence noted. Potency estimations were also unaffected by assay time or type of end point employed. The actual amount of heparin needed to inhibit coagulation is altered, however, by such factors as the state of the plasma (fresh or frozen), the aging of plasma at room temperature, the assay temperature, assay time, and type of end point. Small variations in calcium from the stoichiometrical equivalent of the citrate anticoagulant do not affect the heparin requirement. However, smaller amounts of calcium result in a lowered heparin requirement. The conditions specified for an assay must be rigidly adhered to, particularly in regard to treating the standard and unknown exactly alike.

IN THE previous paper (1) there were described the methods employed, variations seen in the character of the clot during formation and at the end point, evaluation of potency, and a comparison of the clotting curves of heparinized beef and sheep plasmas. The data presented here demonstrate the influence of environmental factors. Some general conclusions were given at the 1947 Federation meeting (2) and the data upon which these were based, together with further information, are included in this paper.

It scarcely seems necessary to state that no two batches of plasma react exactly alike. The differences are reflected in the varying amounts of heparin required for inhibition of clotting and in the character of the whole or partial clots. However, the estimated potency of heparin as determined by comparison with a standard has been found to remain unaltered. Different samples of heparin may vary in purity and potency but there is no reason for believing that commercial heparins differ in their chemical structure and we would thus expect parallel reactions in all plasmas.

The plasmas employed, beef and sheep, were prepared as previously described.[1] Some were

* Received December 2, 1947, from the Department of Pharmacology, St. Louis University School of Medicine, St. Louis 4, Mo.

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‡ Acknowledgment is herewith made to Hoffman-La-Roche, Inc., Nutley, N. J., The Connaught Laboratories, Toronto, and the Wilson Laboratories, Chicago, for the specimens of heparin used.

[1] All plasmas used for obtaining the present information were "slow frozen" by placing the bottles in a General Electric deep freezer, except for the experiments illustrated in Figs. 1 and 2. Plasmas for Expts. 8 and 21 were "quick frozen" in a dry ice-acetone bath and for Expt. 9 "slow frozen." The latter were stored in a box containing dry ice.

employed in the fresh state and others were frozen and stored at about −25°. For use the frozen plasmas were thawed by immersion in the 37° water bath with frequent rotation of the bottle. They were strained through glass wool. Some specimens, especially among many samples of sheep plasma, had flocculent particles not seen in the original plasma. In most cases the flocculent particles "melted" to a transparent state and then dissolved or dispersed during the rise in temperature. Resolution of the particles nearly always occurred in beef plasma but sometimes an appreciable amount of the precipitate in a sheep plasma did not redissolve.

In assessing the influence of the several factors the distinction must be made between potency, meaning the strength of heparin as compared to a standard tested simultaneously, and the dose or concentration, meaning the actual amount of heparin required to inhibit coagulation. In Part I there are given data demonstrating the lack of effect of environmental factors on potency and in Part II the influence of these factors in causing a variation in the amount of heparin required. In some cases the same experiment provided information for both assessments.

PART I. THE STUDY OF VARIOUS FACTORS IN RELATION TO POTENCY

Comparison of Fresh and Frozen Beef and Sheep Plasmas.—Frozen plasma offers several advantages over fresh plasma. It can be prepared at any convenient time in advance of the assays and in sufficient quantity for many tests. Plasmas from several animals may be pooled so a large uniform batch is available. The behavior is established in the first experiment and this experience is available for the future tests on the batch. With fresh plasma the clotting range must be determined each time. The use of frozen plasma has previously been reported by Kuizenga, Nelson, and Cartland (3) and by Wolfrom and McNeely (4), but they made no comparison with fresh plasma. In Table I there are listed the data obtained in assays on the same lots of fresh and frozen plasmas. Blood for these tests was collected during the latter part of July and early August.

The results demonstrate no significant differences in potency when beef or sheep plasma is employed in either the fresh or the frozen state. Except in one case the P values all indicate a high degree of significance to the agreement. In the one case of divergence, the assay of heparin No. 3 on fresh and frozen sheep plasma, the low value for P, 0.02, is due not only to the difference between the means but also to the very low standard error in Expt. 67, namely, 0.2%. The same comparison made in Table II resulted in a P value of 0.48. All other values for the same sample agree statistically.

In assays using fresh sheep plasma more difficulty was encountered in determining the heparin concentration range than in using fresh beef plasma. This may have been due partly to a slower clotting reaction, for density measurements with the electrophotometer suggest this, but more likely it was largely due to the much lower density of clots from sheep plasma. In an experiment with fresh sheep plasma preceding Expt. 67, the concentration range was higher than indicated by the preliminary test and in Expt. 67 illustrated in Table I heparin No. 4 fell at the extreme edge of the range and accurate estimation of the CC_{50}[2] was not possible. This difficulty was considerably less with beef plasma or with either plasma in the frozen state.

Table II gives data obtained in earlier experiments performed on plasma from blood collected in January. These results are in fair agreement with those of Table I. In the series on fresh beef plasma, two of the results gave abnormally high standard errors; however, on each of these only two series of tubes could be used for the estimation rather than the usual four.

Tables I and II both show that even disregarding the three abnormally high standard errors obtained on beef plasma there was still a tendency for slightly better uniformity in assays made on either fresh or frozen sheep plasma. The magnitude of this better uniformity is not great, however.

The results in the two tables also indicate no difference in estimated potencies when winter and summer plasmas are employed or when the assays are carried out at a high room temperature.

Assay Time and Type of End Point.—The actual assay time evidently has no influence on potency provided the standard and unknown are read at corresponding times. This is shown in Table III where the same tubes were read at one hour and three hours. The original Reinert and Winterstein method (6) proposed four hours. In the previous paper (1) a one-hour observation time was suggested. However, when a large amount of material is being tested or if an individual is working alone one hour may be insufficient to prepare all tubes before the first ones must be read. The results of Table I are all based on a two-hour assay; those in Table II on a one-hour assay.

The two end points compared were the all-or-none and the dose-response. The all-or-none end point represents that amount of heparin which inhibits coagulation to such an extent that the plasma will just flow when the tube is tilted carefully without tapping. The dose-response end point represents the amount of heparin which allows the formation of a 50% clot. The terms used to represent these are respectively CC_{0+}, meaning a "clotting concentration" of heparin which permits the formation of slightly more than zero clot, and CC_{50}, meaning the concentration resulting in a 50% clot.[2]

With sheep plasma both end points could be read on the same tubes (Expt. 52). The scatter in the actual potencies obtained is small and the standard errors are all consistently small. The P values

[2] CC_{50} refers to "50% clotting concentration," that is, the amount of heparin that will allow the formation of a 50% clot. In the previous paper the term CD_{50}, meaning "50% clotting dose," was used. It seems more appropriate to refer to "50% clotting concentration" as in (5) since an *in vitro* system is employed. The estimation of the degree of clot formation by visual inspection is a practical means of arriving at a definite end point and it has been considered that it represents a definite proportion of the entire amount of fibrin that may be formed. The extent of conformity to this presumption is under investigation.

TABLE I.—COMPARISON OF FRESH AND FROZEN BEEF AND SHEEP PLASMAS

Plasmas prepared in summer.
Potencies expressed in Provisional International Units per milligram of heparin.
Toronto heparin (137 U/mg.) used as standard. (Potency determined by checking against the Provisional International Standard stated to contain 130 U/mg.)
Under Plasma Column, B = Beef, S = Sheep.

	Heparin Sample	1			2			3			4			
Expt.	Plasma	U/Mg.	S_E, %	*P*	U/Mg.	S_E, %	*P*	U/Mg.	S_E, %	*P*	U/Mg.	S_E, %	*P*	*N*
64	BQ-Fresh	100.4	2.2		101.5	0.9		97.6	0.7		91.4	1.9		4
				0.51			0.38			0.44			0.16	
65	BQ-Frozen	98.5	2.8		99.5	2.7		96.0	2.0		87.2	5.0		4
67	SO-Fresh	98.5	1.1		102.9	0.4		95.1	0.2		<90.0	...	...	4
				0.19			0.85			0.02				
68	SO-Frozen	101.0	1.2		102.7	0.9		98.9	1.0		88.0	0.8	...	4
65–68	Comparison of frozen plasmas	...	...	0.30	...	...	0.19	...	...	0.21	...	...	0.74	...

Frozen plasmas were tested 24 hours after preparation.
S_E = standard error of the mean.
P = probability of agreement.
Assay temperature: 37°. Assay time: 2 hours.
Room temperatures: Expt. 64, 32°; Expt. 65, 27°; Expt. 67 and 68, 34° to 36°.

TABLE II.—COMPARISON OF FRESH AND FROZEN BEEF AND SHEEP PLASMAS

Plasmas prepared in winter.
Potencies expressed in Provisional International Units per milligram of heparin.
Toronto heparin (137 U/mg.) used as standard.

	Heparin Sample	1			2			3			
Expt.	Plasma	U/Mg.	S_E, %	*P*	U/Mg.	S_E, %	*P*	U/Mg.	S_E, %	*P*	*N*
37	BJ-Fresh	105.0	10.4		104.2	1.2		99.9	5.8		2
				0.14			0.25			0.53	
40	BJ-Frozen	98.9	2.1		101.8	1.1		100.8	0.7		4
				0.16			0.14			0.05	
41	BJ-Frozen	95.1	2.4		105.3	2.2		95.1	3.6		4
38	SF-Fresh	100.2	0.9		105.1	1.1		100.3	0.3		4
				0.31			0.43			0.48	
42	SF-Frozen	98.7	0.3		103.3	1.8		99.8	0.3		3
40–42	Comparison of frozen plasmas	...	...	0.93	...	...	0.44	...	...	0.46	.
41–42	Comparison of frozen plasmas	...	...	0.12	...	...	0.48	...	...	0.12	.

Frozen beef plasma tested 7 days after preparation.
Frozen sheep plasmas tested 12 days after preparation.
Assay temperature: 37°; assay time 1 hour.
Room temperature: 22° to 25°.

were not calculated, but by inspection one would expect to find them about the same as those in Tables I and II.

With beef plasma the two end points could not be read on the same series of tubes so two separate assays were made (Expt. 49 and 50). The spread in the estimated potencies is no greater than those estimated on sheep plasma or than the potencies given in the first two tables. By both end points the standard errors are higher than with sheep plasma. The errors with the all-or-none end point are higher in two of the three examples than are those by the dose-response end point. Although this is in accord with theoretical consideration of the nature of the two end points and the manner of determining them, practically, it does not seem to matter a great deal since with either plasma or with either end point the errors are lower than the assay errors

cited by other reports in the literature. In almost any work with biological material even for bioassays a standard error of 5% is considered not excessive. The *in vitro* assay of heparin is more akin to a chemical titration than to a "bio" assay and lower standard errors should be expected, but the vagaries of the very labile plasma make one suspect it still behaves as a "living" substance.

The all-or-none end point was used by Kuizenga, *et al.* (3), for plasma and by most other workers using whole blood. Jaques and Charles (7) were able to obtain an approximate dose-response end point with blood.

At this point it should be mentioned that a minor modification in procedure has recently been introduced which helps reduce the errors slightly. When several unknowns are being tested at the same time an extra set of standard tubes is run at the beginning of the first series. The standard is also run at the end of each series. The results on the standard at the beginning and end of the first series are averaged for calculating the potencies of the intervening unknowns. The standard at the end of the first series also serves as the standard for the beginning of the second series and is averaged with the standard at the end of this series, and so on through the four series of tubes. All potencies in Tables I to III were estimated by this technique except in Expts. 37 and 38 of Table II. In one experiment that was checked the standard errors were about 0.3% lower by this method than by calculating the results on the basis of only the standards at the ends of the series. This difference is hardly consequential but because of the gradual change of plasma (see Part II) it seems preferable to employ the standard in duplicate for each series, which is made possible merely by adding one extra set of five tubes at the beginning of the assay.

PART II. INFLUENCE OF THE VARIOUS FACTORS ON THE AMOUNT OF HEPARIN REQUIRED FOR INHIBITION

Comparison of Fresh and Frozen Plasma.—Tests were conducted on fresh plasma immediately after centrifugation. A portion of the same plasma was placed in the freezer and tested the next day or several days later. Storage time in the frozen state did not seem to be a significant factor although no systematic study of this was made. Taylor, *et al.* (8), have shown that with frozen human citrated plasma the clotting elements remain substantially unchanged for six months, except prothrombin, which is still present in effective quantity (40% of original).

The major changes evidently occur before or during the initial process of freezing and the subsequent thawing. Taylor, *et al.*, also showed that slow thawing (overnight) caused a rather prominent decrease in prothrombin in human plasma.

Table IV shows the comparison between the fresh and frozen plasmas. In every case frozen plasma required more heparin than the original fresh plasma. Frozen sheep plasma required only slightly more heparin but frozen beef plasma required considerably more, the increases averaging about 10% and 70%, respectively.

The data of Table IV also illustrate both the species and individual differences. In the frozen state, sheep plasma required about 75% more heparin than beef plasma while in the fresh state about 150% more. Comparison between individual cases shows a wide spread in the sheep-beef heparin concentration ratio, e.g., 1.14, 1.49, 2.07, and 2.72 for the four possible ratios among the frozen beef and sheep plasmas using heparin No. 1 as the example; that is, in one case the beef and sheep plasmas required almost the same amount of heparin, but in another case the sheep plasma required nearly three times as much.

Effect of Aging After Thawing.—The amount of heparin required to inhibit coagulation is reduced as plasma stands at room temperature after thawing. This is illustrated by data plotted in Fig. 1. Even

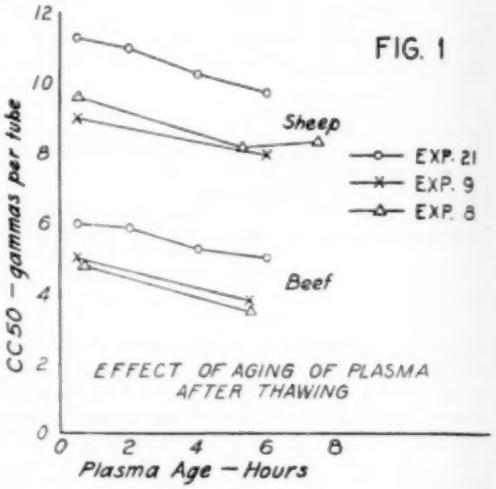

Fig. 1.—The curves show gradually lowered heparin requirements over a period of hours. Aging took place at room temperature.

in regular assays the CC_{50} may go down slightly during the relatively short time required for filling all series of tubes. Picking a few cases at random from different experiments on both plasmas the variations in dosage were:

First test:	3.4	5.2	6.0	3.9	7.9	7.7	5.4
Fourth test:	3.5	5.2	5.7	3.6	7.4	7.1	4.6

The time intervals for preparing the series of tubes from which these values were obtained ran from about three-fourths to one and one-half hours. Intervals as long as the latter did not consistently result in a decrease in the CC_{50} since technical errors or other factors can play a big role in such slight variations. Nevertheless the trend was unmistakable from the above values and from Fig. 1. They indicate the desirability of duplicating the standard heparin for each series as described in Part I, except when no more than one or two unknowns are being run.

The change in the plasma is probably explained by the loss of fibrinogen on standing. Using the electrophotometer the density of clots from unheparinized plasma was shown to be considerably decreased after a few hours' standing. Lavergne

TABLE III.—COMPARISON OF DIFFERENT END POINTS AND OBSERVATION TIMES

Frozen plasmas employed.
Potencies expressed in Provisional International Units per milligram.
Standard: see below.

Heparin Sample				1		2		3		4		
Expt.	Plasma	Obs. time, hours	End Point	U/Mg.	SE, %	U/Mg.	SE, %	Mg.	SE, %	U/Mg.	SE, %	N
49	BI	1	CC_{0+}	98.0	4.7	103.0	2.7	100.3	5.1	..	...	4
49	BI	3	CC_{0+}	95.4	6.2	102.3	1.7	102.8	4.5	..	...	4
52	SF	1	CC_{0+}	(100)[a]	...	99.8	0.7	95.5	0.8	90.5	0.8	4
52	SF	3	CC_{0+}	(100)[a]	...	101.2	0.8	97.0	1.2	90.8	0.7	4
50	BI	1	CC_{50}	(100)[a]	...	97.6	2.8	101.0	2.3	94.6	1.4	4
50	BI	3	CC_{50}	(100)[a]	...	101.7	3.0	102.0	2.5	95.0	1.1	4
52	SF	1	CC_{50}	(100)[a]	...	103.1	0.4	98.4	0.6	93.9	1.3	4
52	SF	3	CC_{50}	(100)[a]	...	102.1	0.3	97.7	0.8	92.2	0.8	4

[a] Heparin No. 1 used as standard. In Expt. 49 Toronto heparin (137 U/mg.) was used as standard.
CC_{0+} = all-or-none end point; slight amount of fibrin present and plasma just flows when tube is tilted.
CC_{50} = dose-response end point; 50% fibrin present (estimated).

TABLE IV.—INFLUENCE OF SPECIES AND STATE OF PLASMA ON CONCENTRATION OF HEPARIN REQUIRED

One-hour observation time.

Heparin Sample			1		2		3		4		5*		
			CC_{50}		CC_{50}		CC_{50}		CC_{50}		CC_{50}		Average
Expt.	Plasma	N	γ	Ratio	γ	Ratio	γ	Ratio	γ	Ratio	γ	Ratio	Ratio
65	BQ-Frozen	4	5.1		5.0		5.2		5.7		3.6		
				1.50		1.52		1.48		1.54		1.44	1.50
64	BQ-Fresh-	4	3.4		3.3		3.5		3.7		2.5		
41	BJ-Frozen	4	2.8		2.5		2.8		...		1.9		
				1.87		1.92		2.00				1.90	1.92
37	BJ-Fresh	2	1.5		1.3		1.4		...		1.0		
68	SO-Frozen	4	7.6		7.5		7.8		8.7		5.8		
				1.06		1.07		1.04		>1.11		1.11	1.07
67	SO-Fresh	4	7.2		7.0		7.5		>7.8		5.2		
42	SF-Frozen	3	5.8		5.5		5.8		...		4.1		
				1.11		1.10		1.11				1.08	1.10
38	SF-Fresh	4	5.2		5.0		5.2		...		3.8		
Average: Frozen Sheep			6.7		6.5		6.8		...		4.95		
				1.74		1.73		1.70				1.80	1.74
Average: Frozen Beef			3.85		3.75		4.0		...		2.75		
Average: Fresh Sheep			6.2		6.0		6.35		...		4.5		
				2.53		2.61		2.70				2.57	2.60
Average: Fresh Beef			2.45		2.3		2.45		...		1.75		

CC_{50} = 50% clotting concentration of heparin in gammas per tube (= 2 cc. solution ≏ 1 cc. pf plasma).
* Toronto heparin.

and Lavergne-Poindessault (9) demonstrated a loss of fibrinogen but no loss of prothrombin in human plasma allowed to stand twenty-four hours at 0°. Taylor, *et al.* (8), could demonstrate little or no fibrinogen in human plasma stored at room temperature for one to six months, although 2% prothrombin was present even after six months.

Effect of Assay Temperature, End Point, and Observation Time.—An experiment was set up to study these factors simultaneously. For the different observation times, one and three hours, the same tubes were observed. With sheep plasma it was also possible to observe both end points in the same series of tubes. With beef plasma the CC_{0+} could not be observed in the same series of tubes from which the CC_{50} was estimated because of the great difference in dosage ranges. Separate series were set up to be run at room temperature and in the water bath at 37°.

The difference in dosage ranges for the CC_{50} and CC_{0+} with beef plasma may be described as follows. The main part of the clotting curve is quite parallel to the curve for sheep plasma. However, as the heparin concentration is increased and the lower portion of the curve reached the curve begins to flatten out. This is illustrated in the figure in the

previous paper (1). With the beef plasmas here used small amounts of coagulum were seen to persist beyond all proportion to the increasing amounts of heparin added. While these persistent "traces" of coagulum have no influence on the CC_{50} they have a marked effect on the CC_{0+} when they form a pellicle at or near the surface to prevent flow on tilting. Beef plasmas apparently vary considerably in their tendency to produce these persistent traces of clot as is evident by comparing the CC_{0+} values in Table V with the published curve. This curve, for beef plasma, reaches the base line (zero clot) with the heparin concentration about 15% above the CC_{50}. In Table V the CC_{0+} figures are 150% higher than the CC_{50}'s. The difference seen between the two end points had no significant effect on the potency estimation as has been mentioned.

heparin which would produce clotting within a specified time. This technique was previously used by the author (5). In assays then, the amounts of calcium were always suboptimal so that the response of the plasma was affected by volumetric errors in measuring the calcium chloride as well as by those in measuring the heparin and plasma itself. Such errors, from these three sources, are not merely dilution errors as would be with the saline solution, but they occur at the critical range of the clotting reaction and may profoundly affect the reaction. It therefore seems more logical to recalcify with an amount of calcium stoichiometrically equivalent to the sodium citrate present. Within limits well within those beyond which volumetric errors of calcium could seriously throw off the results, supra-optimal amounts of calcium have little effect on the coagulation. This is because recalcification with

TABLE V.—INFLUENCE OF TEMPERATURE, OBSERVATION TIME, AND END POINT ON CONCENTRATION OF HEPARIN REQUIRED

Frozen plasmas employed.
Heparin No. 1 used for all tests.

Expt.	Plasma	End Point	37° Observation times 1 hour		37° Observation times 3 hours		22–25° Observation times 1 hour		22–25° Observation times 3 hours		N
			γ	γ	γ	γ	γ	γ	γ	γ	
46	BF	CC_{50}	3.8	3.8	3.9	3.8	3.9	3.9	4.2	4.1	4
46	BF	CC_{0+}	>5.1		>5.1		>5.1		>5.1		4
49	BI*	CC_{0+}	9.4		9.4		...		...		4
45	SE	CC_{50}	5.8	5.8	6.1	6.1	5.5	5.5	5.9	5.9	4
45	SE	CC_{0+}	6.3	6.3	6.5	6.5	5.9	6.0	6.5	6.4	4

* Plasma BI was substituted since the supply of BF was used up. The CC_{50} of heparin No. 1 using plasma BI was 3.7γ at 37° assay temperature and 1 hour observation time. The plasma was thus similar to plasma BF.

In the case of sheep plasmas the CC_{0+} required only a slightly higher dose of heparin than the CC_{50}. In Table V the greatest difference is 10%. The published curve for sheep plasma reaches the base line at 6% higher concentration. Thus even with sheep plasma the spread in the clotting curve is somewhat greater than previously obtained.

The effect of prolonging the observation time from one hour to three hours was to raise the required amount of heparin, using either end point. The effect was slightly greater with sheep than with beef plasma. For both species the effect of longer observation time in raising the required concentration was greater at room temperature than at 37°. These results reflect the continued growth of the clot. This has been demonstrated by the electrophotometer, most clots continuing to increase in density for twenty-four hours.

The effect of raising the assay temperature from room temperature to 37° is slight though greater on sheep than on beef plasma. Both this and the previous observation suggest that beef plasma clots somewhat faster than sheep plasma. In carrying out assays at the two temperatures the clots of both plasmas were more "readable" at the higher assay temperature.

Effect of Calcium Concentration.—The original method of Reinert and Winterstein (6) specified a test for "standardization of plasma" before assaying heparin. The purpose of the test was to determine the amount of calcium necessary for recalcification in the presence of a standard amount of 2 mg. of calcium chloride provides an excess beyond the amount needed in the clotting reaction.[3]

Tests were made using 1, 2, and 3 mg. of calcium chloride for neutralizing the citrate, the results being illustrated in Fig. 2. The plasmas used for these tests, as well as all other plasmas in the work, contained 4 mg. of sodium citrate per milliliter,[4] and the amount of calcium chloride stoichiometrically equivalent to this is approximately 2 mg. (theoretical 1.865 mg.). The amount of heparin required was twice that when only 1 mg. of calcium chloride was used, but there was no difference in heparin requirements between 2 and 3 mg. of calcium chloride.

With some plasmas 1 mg. of calcium chloride was apparently close to sufficient for recalcification since some of the clotting curves were very steep. Below 1 mg. there was obtained a rapid diminution in coagulability as the amount was reduced. Concentrations between 2 and 3 mg. were not tried but it hardly seems likely that any modification of the curves of Fig. 2 would be found.

It may be remarked that in assays conducted over

[3] Two milligrams of calcium chloride per milliliter of citrated plasma was the concentration used by Kuizenga, *et al.* (3).

[4] In collecting blood conditions generally necessitate having the slaughter-house butcher fill the bottles and he usually does not succeed in filling just to the mark. Over-filling is more likely. As much as 15% excess blood, reducing the sodium citrate concentration to 3.5 mg./ml., has caused no noticeable effect on results. The filling error is seldom over 5%, however.

several years the amount of calcium chloride varied from about 0.7 to 1.2 mg. with different fresh beef plasmas. Since changing to 2 mg. for recalcification the standard errors have been rather consistently lower.

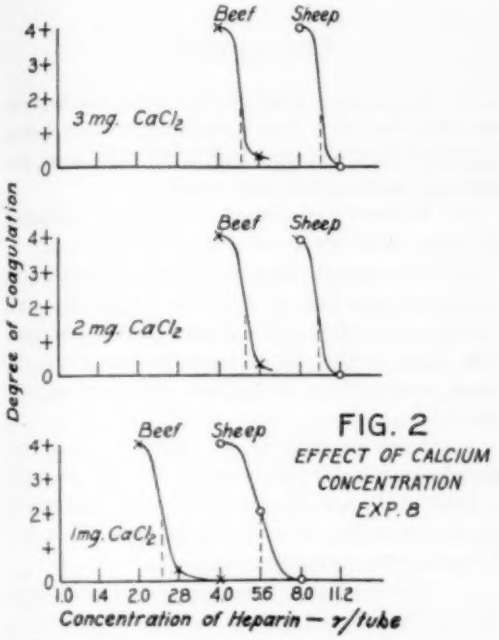

FIG. 2
EFFECT OF CALCIUM CONCENTRATION
EXP. 8

Fig. 2.—Influence of re-calcification with different amounts of calcium chloride on the CC_{50} of heparin. The 50% points (2+) are indicated by dotted lines.

DISCUSSION

The work discussed in this and the preceding papers (1, 2) was undertaken to aid the Committee on Revision of the Pharmacopœia of the United States in setting up an official assay method for heparin. An official method has not yet been decided on but will be following a collaborative study program set up by the Committee on Revision. These particular experiments were designed primarily to compare the use of beef plasma and sheep plasma and were extended to show the effects of various environmental factors.

The results of Part I demonstrate that the potencies of several samples of heparin, estimated by comparing with a standard run concurrently, do not vary with the conditions employed here. The selection of plasma rather than whole blood for the assay medium seems to be particularly that of practicality. It is apparent that for control purposes it is a great advantage to have on hand a large stock of suitable material readily available for use. Stored frozen plasma meets this need. Several liters may be mixed and frozen at one time so that a uniform batch with known clotting characteristics is available. In addition there is a considerable saving in time.

Jalling, Jorpes, and Linden (10) have discussed the use of various clotting systems, including whole blood. They point out that heparin may be partly neutralized by reacting with prothrombin, thrombin, and thromboplastin and that any method which involves this neutralization by only one of these constituents entails some risk. They object to citrated or oxalated plasma (which contains the above three elements) only on the grounds that an excess of calcium ions seriously disturbs the coagulation. This objection has been met by the results illustrated in Fig. 2, which showed that an excess of calcium of at least 50% caused no alteration in the concentration of heparin required. In conclusion they recommend fresh whole blood.

It is possible that different samples of heparin may react differently with the different constituents of the blood and the synthetic anticoagulants in comparison with heparin have been shown to have a different action on plasma in contrast to that on blood (11). Also Jaques and Charles (7) showed differences in relative activity of heparin samples assayed on whole blood and on plasma.

Although these objections may be valid they do not appear too important to the author when it comes to the problem of pharmaceutical control. Although beef heparin has been purified to different degrees there is no evidence that any commercial heparins behave differently from each other in their fundamental reactions.

The development of synthetic anticoagulants and their assay is of no immediate concern to the problem of an official assay method for heparin. Nor is it necessary that a single assay method meet the needs of all types of anticoagulants.

We may summarize the advantages of plasma (beef or sheep) as follows:

1. A large amount of uniform plasma (mixture of several batches) may be kept on hand in the frozen state. This is a practical time-saving point.
2. Once tested, the clotting range of this plasma is known and preliminary tests need not be run. Results can be more easily verified.
3. The degree of coagulation is more easily and more accurately estimated on plasma than on whole blood.
4. Plasma volumes can be measured more accurately and more quickly than blood volumes.
5. Plasma has already been proved to be a satisfactory assay medium and is practically exclusively used by all manufacturers in this country.
6. On occasion frozen plasma can be shipped while fresh blood deteriorates too rapidly.

The influence of the various factors on the actual concentration of heparin required demands less attention than their possible effect on the potency determinations on account of the use of a standard. Nevertheless, they are important in evaluating and planning the technique of the assay since it is important to know how the heparin concentration range may be shifted and, of course, those conditions must be selected which would tend to cause the least variation during the progress of the assay. Most of these factors need little discussion beyond that given in Part II with the results.

Data showing the effect of season were not tabulated as they were deemed too inconclusive to present in organized form. There was no seasonal influence on potency but there was an apparent seasonal effect on the concentration of heparin re-

quired. Since there may be marked individual variations the seasonal trend was not clearcut. There was a tendency for both beef and sheep plasmas to require the most heparin during spring and the least in midwinter. Jorpes and his co-workers (10) found the greatest requirement in summer and the least in winter. The heparin concentration range they found for whole blood was about fourfold, whereas in this work the extremes for any two individual plasmas was only slightly more than twofold for both beef and sheep. It was also noted that winter plasma, particularly beef, clots more homogeneously than that obtained during other seasons.

In the previous paper the statement was made that "a fully clotted sample is one which is as dense as the clot in a tube containing no heparin." This is not strictly correct, for by electrophotometer measurements it was noted that clots from unheparinized plasma were less dense than those containing subminimal amounts of heparin. This can be seen visually but neither the significance nor degree was appreciated prior to the measurements on optical density. The clots of greatest density were in those tubes in which full coagulation, even though delayed, finally occurred. A report on this is to be made elsewhere.

A word is necessary to emphasize that the significance of statistical measurement should not be overrated. The standard errors represent "titration" errors only in the group of solutions as used at the time. They cannot correctly be construed to signify that the true potency is within the limits implied by the standard error. The errors are of course magnified beyond the range seen in ordinary chemical titrations because of the nature of the clotting reaction. The example, in Table I, Expts. 67 and 68, using fresh and frozen sheep plasma, where the potency of heparin No. 3 was estimated as 95.1 U/mg. (Provisional International Units per milligram) and 98.9 U/mg., respectively, serves to illustrate. The P value 0.02 showed these results to be significantly different. The standard errors were 0.2 per cent and 1.0 per cent, respectively. The same solution was used for both tests. In Table II, Expts. 38 and 42, the same sample of heparin but a different solution, also tested on both fresh and frozen sheep plasma, was found to contain 100.3 U/mg. and 99.7 U/mg., respectively, with standard errors of 0.3 per cent and 0.3 per cent and a P value of 0.48 indicating excellent agreement between the results. The P value of 0.02 in the former case indicates a 50 to 1 chance that the two results are significantly different. Since the earlier comparison indicated very little difference, and this in the opposite direction, we must conclude that some unknown factor or factors caused the apparent discrepancy. The comment of Wallace and Snedecor (12) on correlation should be recalled, substituting "statistical" for "correlation" to cover this as well as all other fields: "Unless hard thinking and common sense are used in interpretation, statistical work may do more harm than good."

CONCLUSIONS

1. In assaying commercial samples of heparin no difference in potency was found when tested on either fresh or frozen beef or sheep plasma, nor was any seasonal influence noted.

2. Potency estimations were also unaffected by assay time or type of end-point employed.

3. The actual amount of heparin needed to inhibit coagulation is however altered by such factors as the state of the plasma (fresh or frozen), the aging of plasma at room temperature, the assay temperature, assay time and type of end-point.

4. Small variations in calcium from the stoichiometrical equivalent of the citrate anticoagulant do not affect the heparin requirement. However smaller amounts of calcium result in a lowered heparin requirement.

5. The conditions specified for an assay must be rigidly adhered to, particularly in regard to treating the standard and unknown exactly alike.

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A Pharmacological Study of *dl*-Desoxyephedrine Hydrochloride*

By THOMAS J. HALEY†,‡

The LD_{50} (intraperitoneal) of *dl*-desoxyephedrine hydrochloride for rats and mice was determined to be 54.48 and 61.16 mg./Kg., respectively. The oral LD_{50} for mice was 143 mg./Kg. Chronic toxicity data following intraperitoneal injections in rats are reported. Effects of the drug on the isolated uteri and on the isolated ileum of both mice and guinea pigs were determined. The drug counteracted both pilocarpine and barium spasm of the intestine of these animals. Body temperature was elevated by small doses of *dl*-desoxyephedrine. The compound usually stimulated respiration and caused hypotension in anesthetized rabbits. In dogs the pressor effect was only about $^1/_{100}$ that of epinephrine.

In a recent review of the literature of desoxyephedrine, Haley (1) pointed out that, although the dextro isomer had been extensively studied, little was known concerning the racemic form of the drug. Inasmuch as clinical usage of the racemate had recently commenced, it was thought that a pharmacological and toxicological study might reveal some definite information about the mode of action of the drug.

Graham, Cartland, and Woodruff (2), using the U. S. P. dog assay, found that in pressor activity *dl*-desoxyephedrine hydrochloride was only $^1/_{275}$ as potent as epinephrine. These investigators found that the drug had a dilator effect upon the isolated perfused rabbit lung preparation. Lands, Nash, Granger, and Dertinger (3) found that in dogs the pressor effect of the drug was $^1/_{200}$ that of epinephrine. Swanson, Scott, Lee, and Chen (4) found that in the decerebrated and pithed dog the *dl*-form of the drug was intermediate in pressor activity, with the *l*-isomer showing the greatest pressor effect of the three forms of the drug. However, Hauschild (5) found that the pressor effect in cats was equal when equal doses (0.02 mg./Kg.) of all forms of the drug were administered intravenously. All the above investigators reported that tachyphylaxis developed upon continued administration of the drug. Domenjoz and Fleisch (6) have shown that *dl*- and *d*-desoxyephedrine are about equal in vasoconstrictor activity in cats. Turnbull, Hamilton, Simon, and George (7) found that 0.125 per cent *dl*-desoxyephedrine hydrochloride had a prolonged vasoconstrictor action on the human nasal mucosa. Further, there was no histological evidence of tissue damage. Novelli and Tainter (8) have shown that the *dl*-form is less potent as a central motor stimulant than the *d*-isomer when measured upon rats in the jiggle cage. Graham, *et al.* (2), found that the intravenous LD_{50} of the racemate in large rats was 20 mg./Kg. Lands, *et al.* (3), using a small number of mice found that the approximate intraperitoneal LD_{50} of the *dl*-compound was 70 mg./Kg.

EXPERIMENTAL

Acute Toxicity.—A total of 110 male white rats of the Wistar strain and a total of 98 white mice of both sexes were employed in the determination of the LD_{50} values. The rats weighed between 212 and 428 Gm. (average 310 Gm.). The mice used in the determination of the intraperitoneal toxicity weighed 20 to 40 Gm. (average 27 Gm.) and those used in the determination of the oral toxicity weighed 21 to 36.5 Gm. (average 29 Gm.). The gastric intubation was accomplished with a blunt 18-gauge, $2^1/_2$-inch hypodermic needle; the dose was given in a volume of 0.25 to 0.5 cc. All the animals receiving the same dose were caged together and the room temperature was maintained at approximately 65° F.

The signs of acute toxicity observed after intraperitoneal and oral administration of *dl*-desoxyephedrine hydrochloride in both rats and mice were: piloerection, excitation, gnawing at the bottom of the cage, salivation, nasal hemorrhage, exophthalmos, and S-shaped tail, clonic convulsions, and death by respiratory paralysis. Death usually occurred within two to seven minutes after intraperitoneal injection and within 24 hours after oral administration. The respiratory paralysis seen was central in origin, because the muscle of the diaphragm contracted after direct electrical stimulation and after stimulation of the phrenic nerve. The convulsant action of the drug was shown to be neurotropic and not musculotropic because the S-shaped tail was abolished after sectioning the cord anterior to the base of the tail. Further, the convulsant effect on the hind limbs was also abolished by sectioning the cord anterior to the pelvic girdle. Table I gives the LD_{50} values determined in this study and calculated by the method of Bliss (9).

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TABLE I.—LD_{50} VALUES OF *dl*-DESOXYEPHEDRINE HYDROCHLORIDE IN ANIMALS

Animal	Number Used	Route	Slope	LD_{50}, Mg./Kg.	Standard Error
Rat, large	83	Intraperitoneal	24.84	54.48	±0.83
Mouse	40	Intraperitoneal	44.79	61.16	±0.34
Mouse	25	*Per os*	25.47	143.00	±0.60

TABLE II.—AVERAGE BODY WEIGHT OF RATS UNDER PROLONGED ADMINISTRATION OF *dl*-DESOXYEPHEDRINE HYDROCHLORIDE

Days	Control	Group 1, 0.5 Mg./Day	Group 2, 1.0 Mg./Day	Group 3, 1.5 Mg./Day	Group 4, 2.0 Mg./Day	Group 5, 3.0 Mg./Day
	(5)	(5)	(5)	(5)	(5)	(5)
0	223.8 Gm.	375.8 Gm.	246.6 Gm.	376.2 Gm.	248.8 Gm.	380.2 Gm.
	(5)	(4)	(5)	(5)	(5)	(5)
28	235.8 Gm.	351.0 Gm.	258.6 Gm.	351.8 Gm.	242.4 Gm.	351.4 Gm.
	(5)	(3)	(5)	(5)	(5)	(4)
63	248.0 Gm.	346.6 Gm.	275.0 Gm.	338.2 Gm.	237.6 Gm.	325.2 Gm.
	(5)	(3)	(5)	(5)	(4)	(3)
98	262.2 Gm.	349.0 Gm.	269.4 Gm.	307.8 Gm.	237.0 Gm.	295.7 Gm.
Total drug given	0	35.0 mg.	70.0 mg.	105.0 mg.	140.0 mg.	210.0 mg.
Weight gain or loss	+38.4 Gm.	−26.8 Gm.	+22.8 Gm.	−68.4 Gm.	−11.8 Gm.	−90.5 Gm.

Chronic Toxicity.—The effects of continued intraperitoneal administration of the drug in white rats in doses of 0.5, 1.0, 1.5, 2.0, and 3.0 mg. daily for five days of the week were determined over a period of 98 days. Six groups of five animals each were used. The control animals received 0.1 cc. of normal saline solution in place of the drug. The animals were weighed at the beginning of the experiment and each week thereafter. At the end of the period all survivors were sacrificed and their organs examined macroscopically.

The same symptoms of toxicity seen in the acute toxicity study were also observed in this study, except that convulsions were absent. Further, ejaculation was observed in all medicated groups at least three to four times a week. The signs of central stimulation were absent in Group 1 after the 28th day, but were seen in all other groups throughout the study.

Several animals died early in the study, but their deaths were not attributed to the drug, because of the small amount of the drug administered and the normal appearance of the organs at autopsy. One animal in Group 4 died on the 84th day and two animals in Group 5, one on the 56th day and one on the 93rd day. These deaths were due to the drug because postmortal examination showed the following: hemorrhagic lungs, intestines, and urinary bladder; lungs and liver spotted; and acute cardiac hypertrophy. The intestines were much distended with gas and the abdominal cavity gave off a putrid odor. This latter could not have been due to putrefaction because the bodies were still warm and rigor mortis had not set in when the examinations were made.

Autopsy of all the surviving animals on the 98th day showed that outside of the loss of body fat, rough skin, coarse hair, acute cardiac hypertrophy, and a slight enlargement of the adrenals, the animals were no different from the normal controls. The hearts of the medicated animals were approximately twice the size of the controls and most of the damage was in the right ventricle. This damage consisted of bands of fibrous tissue.

Table II shows the effect of continued administration of *dl*-desoxyephedrine hydrochloride on the body weight of the animals studied. The total weight loss was greatest with the larger doses of the drug. Further, the smaller animals had less weight loss than the larger ones. The medicated animals all had a decreased appetite, which could account for part of the weight loss observed. The great central stimulation, which caused excitation and rapid movement about the cage, could account for the balance of the weight loss. The weight gain in Group 2 could be explained on the basis of the age of the animals and on the lesser susceptibility of younger animals to this type of drug.

Uterine Action.—The effects of *dl*-desoxyephedrine hydrochloride on the isolated uteri of mice and guinea pigs were studied by the Magnus method. A bath holding 125 cc. of Locke-Ringer's solution and thermostatically regulated at 37.5 ± 0.1° was used. In five experiments with the guinea pig uterus, the strip was standardized to a definite height of contraction (approximately 75 mm.) using U. S. P. standard posterior pituitary solution.

The mouse uterus (twelve experiments) reacted as follows: Dilutions of 1:1,250,000 and 1:625,000 increased amplitude and decreased rate; 1:125,000 decreased amplitude and rate of contraction. The guinea-pig uterus (seven experiments) reacted as follows: Dilution of 1:1,250,000 increased amplitude and decreased rate and 1:625,000 and 1:125,000 decreased amplitude and increased rate of contraction. From these results it must be concluded that small doses of the drug are stimulatory and larger doses are inhibitory to the isolated uterus of both animals.

Intestinal Action.—The effects of the drug on the isolated ileum of mice, guinea pigs, and rabbits were studied by the Magnus method using Sollmann-Raedmaeker's solution in the same bath used for the uterine experiments. The action was studied alone

and after either pilocarpine hydrochloride or barium chloride spasm.

The mouse ileum (eight experiments) responded to dilutions of 1:1,250,000, 1:625,000, 1:250,000, and 1:125,000 of *dl*-desoxyephedrine with a decrease in amplitude and rate of contraction. The guinea-pig ileum (ten experiments) responded as follows: 1:1,250,000, 1:625,000, and 1:25,000 decreased amplitude and increased rate; 1:250,000 increased amplitude and rate; 1:125,000 increased amplitude and decreased rate, and 1:12,500 both amplitude and rate of contraction decreased. The rabbit ileum (ten experiments) responded as follows: 1:1,250,000 and 1:125,000 no change in amplitude or rate; 1:62,500 decreased amplitude and increased rate; and 1:25,000, 1:12,500, and 1:6250 decreased amplitude and rate of contraction. In all these experiments the rhythm of contraction was unchanged. In several cases, guinea-pig and rabbit ileum gave a typical sympathomimetic relaxation but it was not as pronounced or as prolonged as that given by epinephrine.

In fourteen experiments it was found that *dl*-desoxyephedrine hydrochloride in a concentration of 1:625,000 counteracted the spasm of mouse intestine produced by the addition of 1 cc. of 1:1000 of pilocarpine hydrochloride to the bath. The addition of a second dose of pilocarpine solution was without effect. In thirteen experiments using guinea-pig intestine the pilocarpine spasm was counteracted by *dl*-desoxyephedrine in a concentration of 1:6250. Here also a second dose of pilocarpine was without effect. In fourteen experiments using rabbit intestine the results were the same as with the guinea-pig intestine.

In fourteen experiments using mouse intestine, *dl*-desoxyephedrine in a concentration of 1:625,000 counteracted the effect of 1 cc. of 1:1000 barium chloride solution. A second dose of barium chloride was not effective. In six experiments barium chloride did not cause a spasm. In both the guinea pig (thirteen experiments) and the rabbit (ten experiments), *dl*-desoxyephedrine in a concentration of 1:6250 counteracted the effect of 1 cc. of 1:1000 barium chloride. In three experiments (rabbit intestine) 1:12,500 of *dl*-desoxyephedrine counteracted the barium spasm. In all cases the second dose of barium was ineffective after desoxyephedrine. However, in two experiments the rabbit intestine did not respond to barium chloride.

Effect on Body Temperature.—Five rabbits (2.66 to 3.33 Kg. body weight), that had been used for U. S. P. pyrogen testing, were injected intravenously with a standard dose of 1 mg. of *dl*-desoxyephedrine hydrochloride. The dose was given in a total volume of 10 cc. and body temperature was taken rectally at one-hour intervals over a period of five to six hours. At the same time measurements of pupil diameter were taken. In four experiments, using the same group of five animals, the temperature rise in all animals was from 0.2 to 3.6° F. (average 1.8° F.). This hyperthermia persisted over a period of four hours whereas no sign of central stimulation or mydriasis was observed.

Effect on Blood Pressure and Respiration.—Six rabbits, weighing 4 to 5 Kg., were anesthetized with sodium pentobarbital (20 mg./Kg.) and urethane (1.5 cc./Kg. of 20 per cent) given intravenously and intraperitoneally, respectively. Blood pressure was recorded from the carotid artery with a mercury manometer, respiration with a valve system connected to the trachea, and all injections were given directly into the ear vein. All injections of the drug are expressed as the total amount administered and not as mg./Kg. However, when calculated on a microgram/kilogram basis they are in the range of dosage used by Hauschild (5). In two experiments the animals were not atropinized in order to observe any cardiac side effects which might be due to the vagus nerve. These animals died of respiratory paralysis followed by cardiac standstill 25 minutes after the beginning of the experiment. In both cases a dose of 10 mg. of the drug caused a hypotension which contributed to the fatal outcome. In four atropinized animals (1 to 2 mg. of atropine sulfate), the first dose of 1 mg. of *dl*-desoxyephedrine increased blood pressure 10 to 20 mm. while the second dose of 1 mg. caused a prompt fall of 5 to 15 mm. followed by a rise of 10 to 20 mm. Doses of 5 and 10 mg. usually produced decreases of 15 to 35 mm., sometimes followed by increases up to 30 mm. Tachyphylaxis was observed in that the response to a given dose was not constant. Further, a reversal of the pressor response often occurred after the first dose of the drug. In one animal a dose of 20 mg. of Dibenamine (N,N-dibenzyl-beta-chloroethylamine), a new sympathoadrenal blocking agent, was administered prior to a dose of *dl*-desoxyephedrine. Inasmuch as there was no reversal of the desoxyephedrine pressor response after Dibenamine and because the animals used regularly gave a pressor reversal after *dl*-desoxyephedrine itself further use of Dibenamine was abandoned. It is possible that if cats or dogs had been used the Dibenamine would have given results similar to those reported by Nickerson and Goodman (10) for epinephrine. In general respiration was decreased in volume and increased in rate following desoxyephedrine administration regardless of the dose given.

A comparison of the pressor response of epinephrine and *dl*-desoxyephedrine was made in two dogs under sodium phenobarbital anesthesia (175 mg./Kg. intraperitoneally). It was found that *dl*-desoxyephedrine was about $^1/_{500}$ as potent as epinephrine. Tachyphylaxis occurred with both animals and hypotension was observed in one animal after a dose of 10 mg.

SUMMARY

1. The LD_{50} values of *dl*-desoxyephedrine hydrochloride by intraperitoneal injection in rats and mice and by oral administration in mice were determined.

2. The signs of both acute and chronic toxicity due to the drug are described and it was shown that the convulsant action is neurotropic and not musculotropic.

3. The most pronounced effect of chronic intoxication in rats was acute cardiac hypertrophy with the greatest damage being found in the right ventricle. Body weight was also affected, the larger animals showing a greater weight loss than the smaller ones. Further, the weight loss was greater with the larger doses.

4. The isolated uteri of mice and guinea pigs were stimulated by low concentrations and inhibited by higher concentrations of the drug.

5. The effects of the drug on the isolated ileum of mice, guinea pigs, and rabbits are described. Also it was shown that the drug counteracts the intestinal spasm caused by both pilocarpine hydrochloride and barium chloride and that those agents were ineffective after the administration of *dl*-desoxyephedrine.

6. *dl*-Desoxyephedrine hydrochloride has a definite hyperthermic action which is independent of its other actions.

7. It has been shown that *dl*-desoxyephedrine hydrochloride has a slight pressor effect in rabbits but in general the hypotensive effects are more pronounced. Respiration was usually decreased in amplitude and increased in rate. When compared with ephinephrine in dogs, *dl*-desoxyephedrine hydrochloride has a pressor value of approximately $^1/_{500}$.

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Reactions With Pentachloroethane. I. Hydrochlorination of Ephedrine*,†

By C. E. MILLER‡

Pentachloroethane has been the subject o investigation to find some application of this chemical to pharmacy. The activity of pentachloroethane toward amines suggested the possibility of using it as a hydrochlorinating agent for alkaloids and related products. It has been demonstrated that pentachloroethane and ephedrine employing 1,4-dioxane as a solvent react to give ephedrine hydrochloride in yields of 80 per cent.

PENTACHLOROETHANE, because of its availability and relative inertness, has enjoyed wide usage as a highly chlorinated solvent and a diluent in certain chemical reactions. From a structural consideration the compound is unique in having only one atom of hydrogen; the other valence bonds of carbon being satisfied by the chlorine atoms present in the molecule. One of the projects at this institution has been the development of some application of this versatile chemical to pharmacy. It is the purpose of this paper to present information obtained relative to the splitting out of hydrogen chloride from pentachloroethane when it is reacted with aromatic hydroxy secondary amines, of which ephedrine may be cited as a specific example. A typical run is described in detail.

EXPERIMENTAL

Sixteen and one-half grams of ephedrine (0.1 mole) and 100 cc. of redistilled 1,4-dioxane were placed in a 500-cc. three-necked flask fitted with a dropping funnel, sealed stirrer, and a reflux condenser. Gentle warming was necessary to effect solution of the ephedrine in 1,4-dioxane. To this solution was added slowly, with stirring, 20.2 Gm. (0.1 mole) of pentachloroethane. After a few minutes the clear solution became turbid, a temperature rise of about 15° was noted and the amount of precipitate increased markedly. After this initial

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temperature rise the flask and contents were heated to the boiling point of 1,4-dioxane for half an hour and then cooled to room temperature. The solid material was collected on a Büchner funnel, washed with 100 cc. of 1,4-dioxane and dried under vacuum. The resulting white needle-shaped crystals thus obtained weighed 15.5 Gm., representing a yield of 70% and, m. p. 218.9°, corrected.

Anal.—Calcd. for $C_{16}H_{16}NOCl$: N, 6.94; Cl, 17.68. Found: N, 6.89; Cl, 17.44.

The reaction proceeds without solvent, but at a somewhat slower rate. In one run using 10% excess of pentachloroethane and no solvent, the maximum precipitation had not occurred after two hours. As further proof of the reaction, there was isolated the expected unsaturated by-product, perchloroethylene, which was identified by boiling point and decolorization of bromine in chloroform.

DISCUSSION

A survey of the literature discloses little information concerning the removal of HCl from polychloroaliphatic compounds containing one atom of hydrogen adjacent to a polyhalogenated carbon atom. Ruggeberg (1) has shown that primary amines split HCl from pentachloroethane. Preliminary experimentation indicates that the more complicated aromatic nitrogenous bodies containing several benzene nuclei react very slowly with pentachloroethane in the absence of catalysts. A future paper will discuss the effect of catalysts on the hydrochlorinating action of pentachloroethane.

CONCLUSIONS

1. It has been demonstrated that ephedrine will remove HCl from pentachloroethane with and without the use of solvent.
2. The potential use of pentachloroethane as a hydrochlorinating agent has been demonstrated.
3. The expected by-product, perchloroethylene, was isolated and identified.

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The Determination of Ephedrine in Various Medicinal Combinations*,†

By W. W. HILTY and D. T. WILSON

Alternate methods for the determination of ephedrine in U. S. P. XIII and N. F. VIII preparations are suggested. Procedures for the separation and determination of ephedrine when found in combination with other medicinals such as barbiturates, acetylsalicylic acid, phenacetin, and aminophylline are described in detail.

THE PRESENT accepted methods for the assay of medicinal preparations in which ephedrine is the active ingredient or is present in combination with other medicinal agents follow two general procedures.

In the first method ephedrine is treated as a volatile alkaloid. The ephedrine base is liberated from its salt by adding an excess of sodium hydroxide and extracting with successive portions of ether. It is finally removed from the ether by extracting it with a standard acid solution. This procedure is now employed as the official U. S. P. XIII and N. F. VI I (3, 4) assay for the salts of ephedrine, tablets, ampules, syrups, solutions, and jellies containing ephedrine.

The second procedure, originally proposed by Hilty (1) and further discussed by Welsh (5), involves preliminary acid digestion and subsequent distillation of the ephedrine and any other basic nitrogenous degredation products from an alkaline medium into an excess of standard acid. This procedure is now employed as the official method of the N. F. VIII for the assay of filled capsules of ephedrine salts and filled capsules of ephedrine sulfate and phenobarbital.

We, in our laboratory, have found it necessary

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to develop methods of analysis for a large number of preparations in which ephedrine is the active ingredient in a wide variety of diluents. We have also been called upon to develop methods of analysis for preparations in which ephedrine is present in combination with other commonly associated medicinals such as barbiturates, acetylsalicylic acid, phenacetin, and aminophylline.

Holt (2) has suggested a method of separation for some of these ingredients based on the difference of their solubilities in various solvents. In our investigations we found that ephedrine salts were not sufficiently insoluble in the common solvents suggested. This resulted in values lower than the theoretical ephedrine content.

In the process of developing these methods, various separations were investigated. However, it seemed quite logical that the U. S. P. XIII general procedures for ephedrine salts might be utilized as a starting point in the separation of some of these mixtures.

The method described by Hilty is applicable to many preparations containing ephedrine as the single active ingredient and is particularly desirable when the total amount of working time is considered.

The following methods described for the salts of ephedrine, tablets, ampuls, solutions, syrups, and jellies of ephedrine and ephedrine spray are suggested as alternates for the methods described in the U. S. P. XIII and N. F. VIII for these various preparations.

In the preparation of this paper it has been considered advisable to describe separately the analysis of each combination in its complete form in order to avoid the possibility of confusion in making cross references.

EXPERIMENTAL

Procedure for Ephedrine Sulfate and Ephedrine Hydrochloride.—Transfer about 0.30 Gm. of ephedrine sulfate or ephedrine hydrochloride, previously dried for two hours at 100° and accurately weighed, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE I—RECOVERY OF EPHEDRINE SALTS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3000	0.3000	....	....
B	0.3000	0.2996	....	....
C	....	....	0.3000	0.2997
D	....	....	0.3000	0.2994

Procedure for Tablets Ephedrine Sulfate and Ephedrine Hydrochloride.—Weigh not less than 20 tablets, reduce them to a fine powder without appreciable loss, and transfer an accurately weighed portion, equivalent to about 0.30 Gm. ephedrine sulfate or ephedrine hydrochloride, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. ephedrine hydrochloride or 0.004285 Gm. ephedrine sulfate.

TABLE II.—RECOVERY OF EPHEDRINE SALTS FROM TABLETS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3240	0.3227	....	....
B	0.3240	0.3216	....	....
C	....	....	0.3240	0.3214
D	....	....	0.3240	0.3235

Procedure for Ampuls and Solutions of Ephedrine Salts.—Transfer an accurately measured portion of the ampul solution, equivalent to about 0.30 Gm. of ephedrine sulfate to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and

a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate or 0.004034 Gm. ephedrine hydrochloride.

TABLE III.—RECOVERY OF EPHEDRINE SALTS FROM AMPULS AND SOLUTIONS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Sulfate, Gm.	Ephedrine Sulfate Recovered, Gm.	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.
A	0.3000	0.2996	....	....
B	0.3000	0.2995	....	....
C	....	....	0.3000	0.2990
D	....	....	0.3000	0.3020
E	0.2500	0.2493	....	....
F	0.2500	0.2500	....	....

Procedure for Ephedrine Jellies.—Transfer an accurately weighed portion of ephedrine jelly, equivalent to about 0.25 Gm. of ephedrine, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE IV.—RECOVERY OF EPHEDRINE FROM JELLIES WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine Gm.	Ephedrine Recovered, Gm.
A	0.2500	0.2522
B	0.2500	0.2527

Procedure for Ephedrine Spray.—Transfer an accurately measured volume of the spray, equivalent to about 0.25 Gm. ephedrine, to a 500-cc. Kjeldahl flask. Add 25 cc. of concentrated hydrochloric acid, and an antibump tube. Heat to boiling and boil under a water-cooled reflux condenser for two hours. Cool and wash down the condenser with 50 cc. distilled water followed by 50 cc. of ether and 50 cc. of petroleum benzin. Mix thoroughly and transfer the contents of the flask to a 500-cc. separatory funnel with the aid of two 25-cc. portions of ether and two 25-cc. portions of distilled water. Wash the aqueous portion with the ether-petroleum benzin mixture to remove the oily constituents and transfer the aqueous portion to a 500-cc. Kjeldahl flask. Wash the ether solution of the oily substances with two 25-cc. portions of distilled water, adding the washings to the aqueous portion in the 500-cc. Kjeldahl flask. Warm the aqueous portion in the flask on a steam bath to remove the last traces of ether. Cool, and add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T.S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.003302 Gm. of anhydrous ephedrine.

TABLE V.—RECOVERY OF EPHEDRINE FROM SPRAYS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine, Gm.	Ephedrine Recovered, Gm.
A	0.2500	0.2490
B	0.2500	0.2540

Procedure for Syrup Ephedrine Sulfate When No Other Nitrogenous Substances Are Present.—Transfer an accurately measured volume of syrup ephedrine sulfate, equivalent to about 0.30 Gm. ephedrine sulfate, to a 500-cc. Kjeldahl flask. Add 40 cc. distilled water, 25 cc. concentrated hydrochloric acid, and an antibump tube. Heat to boiling and continue to boil under a reflux condenser for one and one-half hours. Cool and wash the condenser with 50 cc. distilled water. Add sufficient distilled water to the Kjeldahl flask to make the contents measure approximately 225 cc. Now add 1 Gm. of zinc dust and connect the flask with a condenser and a receiver containing exactly 40 cc. of 0.05 *N* sulfuric acid, add 50 cc. of 1 to 1 sodium hydroxide solution, and distill until about 150 cc. of distillate have been collected. Cool the distillate, if necessary, and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Perform a blank determination with the same quantities of reagents and in the same manner and make any necessary corrections.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate.

TABLE VI.—RECOVERY OF EPHEDRINE SULFATE FROM SYRUPS WHEN ASSAYED BY DISTILLATION METHOD

Sample	Ephedrine, Sulphate Gm.	Ephedrine Sulphate Recovered, Gm.
A	0.1296	0.1292
B	0.1296	0.1310

Procedure for Combination of Ephedrine Salts and a Barbiturate.—Weigh at least 20 tablets and reduce them to a fine powder. Weigh accurately a portion of the powder equivalent to about 0.1 Gm. ephedrine sulfate and transfer completely to a separator and add 10 cc. of 2% sodium hydroxide. Extract with at least six 25-cc. portions of ether, collecting these extracts in a second separator. Wash the combined extracts with three 15-cc. portions of distilled water, or until these washings are no longer alkaline to litmus, collecting these washings in a third separator. Extract this wash water with 25 cc. of ether and add the ether to the combined ether extracts in the second separator. Now to this separator add 15 cc. of 0.05 *N* sulfuric acid and shake thoroughly. After the separation is complete draw the aqueous layer into a beaker. Wash the ether with two portions of distilled water, collecting these washings in the beaker, then warm on a steam bath to remove all traces of ether. Titrate the excess acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator.

Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004285 Gm. ephedrine sulfate.

Combine the water wash in the third separator with the alkaline solution in the first separator. Acidify with hydrochloric acid and extract with five 25-cc. portions of a solvent consisting of one part alcohol and four parts chloroform, collecting these extracts in a second separator. Wash the combined extracts with 25 cc. diluted hydrochloric acid. Filter the chloroformic solution into a tared beaker and evaporate on a steam bath with the aid of a current of air, dry the residue at 100° for fifteen minutes, then cool and weigh as the barbituric acid.

TABLE VII.—RECOVERY OF EPHEDRINE SALTS AND BARBITURATES WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Salt Gm.	Ephedrine Salt Recovered Gm.	Barbiturate Gm.	Barbiturate Recovered Gm.
A	0.0972	0.0984	0.1944	0.1944
B	0.0972	0.0963	0.1944	0.1948
C	0.0972	0.0980	0.1944	0.1952
D	0.0972	0.0968	0.1944	0.1932

Procedure for Filled Capsule Combinations of Ephedrine Hydrochloride, Aminophylline, and 'Amytal' (Iso-amyl Ethyl Barbituric Acid, Lilly).—Weigh not less than 20 capsules and determine the average weight of each. Transfer the contents of a sufficient number of average weight capsules, equivalent to approximately four grains of aminophylline, to a separator. Add 25 cc. of 2% sodium hydroxide and extract with six portions of ether, collecting these extracts in a second separator. Wash the ether extracts with two 10-cc. portions of distilled water and transfer the water to another separator. Extract the wash water with 15 cc. of ether and add the ether to the combined ether extraets and the wash water to the alkaline solution contained in the first separator. Extract the combined ether solutions with 10 cc. of 0.05 *N* sulfuric acid, accurately measured, then with successive portions of 10 cc. and 5 cc. of distilled water. Combine the sulfuric acid and water extracts in a beaker, and warm on a steam bath until the odor of ether is no longer perceptible. Cool the solution and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. ephedrine hydrochloride.

Render the combined alkaline solutions in the first separator acid to litmus by the addition of dilute sulfuric acid and transfer the solution to a 250-cc. beaker with the aid of small portions of distilled water. Make the solution alkaline to litmus paper by the addition of ammonium hydroxide and then add 6 cc. in excess. Add 20 cc. of 0.10 *N* silver nitrate and allow the mixture to stand until the precipitate settles. Filter the mixture through a wetted filter paper and wash the precipitate with several small portions of distilled water until the subsequent filtrate yields no test for silver nitrate. Transfer the filter paper and the precipitate to the original beaker and dissolve the precipitate in 10 cc. of concentrated nitric acid. Add 50 cc. of distilled water, cool, and add 2 cc. of ferric ammonium sulfate T. S., and titrate the residual silver with 0.10 *N* ammonium thiocyanate. Each cc. of 0.10 *N* ammonium thiocyanate is equivalent to 0.0230 Gm. aminophylline.

Transfer the filtrate obtained above to a suitable separator and render it distinctly acid to litmus by the addition of concentrated hydrochloric acid and extract with five portions of a mixture, composed of one part alcohol and four parts chloroform, collecting these extracts in a second separator. Wash the alcohol-chloroform extracts with 25 cc. of 2% hydrochloric acid and filter into a tared beaker and evaporate on a steam bath with the aid of a current of air. Dry the residue at 100° for ten minutes and weigh as 'Amytal'.

Procedure for Filled Capsule Combinations of Ephedrine Hydrochloride, Acetylsalicylic Acid, 'Amytal', and Phenacetin.—Weigh not less than 20 capsules and determine the average weight of each. Transfer the contents of sufficient average weight capsules to yield approximately seven grains of phenacetin to a separator. Add 25 cc. of 2% sodium hydroxide and extract with six portions of ether, collecting these extracts in a second separator. Wash the ether extracts with two 10-cc. portions of distilled water and transfer the water to another separator. Extract the wash water with 15 cc. of ether and add the ether to the combined ether extracts and the wash water to the alkaline solution contained in the first separator. Retain this alkaline solution for the determination of acetylsalicylic acid and 'Amytal' as described later in this method.

Extract the combined ether solutions in the second separator with 10 cc. of 0.05 *N* sulfuric acid, accurately measured, then with successive 10-cc. and 5-cc. portions of distilled water. Combine the acid and water extracts in another separator and wash with a 15-cc. portion of ether. Wash this ether extract with 10 cc. of distilled water, combining the water portion with the acid extracts collected in a beaker and the ether portions with the ether extracts retained in the separator. Warm the acid extracts in the beaker on a steam bath until the odor of ether is no longer perceptible. Cool and titrate the excess of acid with 0.02 *N* sodium hydroxide, using methyl red T. S. as the indicator. Each cc. of 0.02 *N* sulfuric acid is equivalent to 0.004034 Gm. of ephedrine hydrochloride.

TABLE VIII.—RECOVERY OF EPHEDRINE HYDROCHLORIDE, AMINOPHYLLINE, AND 'AMYTAL' WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.	Aminophylline, Gm.	Aminophylline, Recovered Gm.	'Amytal', Gm.	'Amytal' Recovered, Gm.
A	0.0486	0.0486	0.2592	0.2598	0.0486	0.0489
B	0.0486	0.0485	0.2592	0.2552	0.0486	0.0488
C	0.0486	0.0483	0.2592	0.2542	0.0486	0.0489

TABLE IX.—RECOVERY OF EPHEDRINE HYDROCHLORIDE, ACETYLSALICYLIC ACID, 'AMYTAL', AND PHENACETIN WHEN ASSAYED BY PROPOSED METHOD

Sample	Ephedrine Hydrochloride, Gm.	Ephedrine Hydrochloride Recovered, Gm.	Acetylsalicylic Acid, Gm.	Acetylsalicylic Acid Recovered, Gm.	'Amytal', Gm.	'Amytal' Recovered, Gm.	Phenacetin, Gm.	Phenacetin Recovered, Gm.
A	0.0432	0.0420	0.2592	0.2504	0.0972	0.0949	0.4536	0.4335
B	0.0432	0.0416	0.2592	0.2476	0.0972	0.0956	0.4536	0.4454
C	0.0432	0.0416	0.2592	0.2660	0.0972	0.1004	0.4536	0.4492

Transfer the combined ether extracts to a tared beaker and evaporate to dryness on a steam bath. Dry in a desiccator to constant weight and weigh as phenacetin.

Render the combined alkaline solutions in the first separator acid to litmus paper by the addition of hydrochloric acid and extract with five portions of a solvent consisting of one part alcohol and four parts chloroform. Collect these alcohol-chloroform extracts in another separator and wash with 15 cc. of 2% hydrochloric acid and discard the acid portions. Extract the chloroform solution with two 50-cc. portions of 4% sodium bicarbonate solution and then wash with 20 cc. of distilled water. Combine the sodium bicarbonate solution and the wash water and extract with two 25-cc. portions of chloroform, collecting these chloroformic extracts in the separator with the retained chloroform. Filter the chloroform extracts into a tared beaker and evaporate on a steam bath with the aid of a current of air. Dry the residue at 100° for ten minutes, cool, and weigh as 'Amytal'.

Transfer the sodium bicarbonate solution to an Erlenmeyer flask and boil for five minutes. Add 5 cc. of 20% sodium hydroxide and boil for fifteen minutes. Cool and dilute to 500 cc. with distilled water. Transfer an aliquot equivalent to approximately one grain of acetylsalicylic acid to a glass-stoppered flask. Add 25 cc. of 0.10 *N* bromine and 6 cc. hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc. potassium iodide T. S. and titrate the liberated iodine with 0.10 *N* sodium thiosulfate.

Each cc. of 0.10 *N* bromine is equivalent to 0.003 Gm. acetylsalicylic acid.

The acetylsalicylic acid in the sodium bicarbonate solution should be determined as soon as possible to prevent hydrolysis.

CONCLUSIONS

Alternate assay procedures are suggested for some of the U. S. P. XIII and N. F. VIII preparations containing ephedrine alkaloid or an ephedrine salt.

Systematized procedures for the separation and analysis of medicinal preparations containing ephedrine as a common ingredient are described.

Each method is presented in full detail to avoid any possible confusion which may result from the necessity of referring from one method to another for details that might otherwise be omitted.

The results obtained by the authors in making these separations are listed in tables accompanying this article and have been found to be quite satisfactory.

In our opinion these methods provide adequate assay procedures for medicinal preparations of this type.

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A Preliminary Investigation of the Antioxidant Effect of Nordihydroguaiaretic Acid in Cod-Liver Oil*

By O. GISVOLD, F. BOPE, and C. H. ROGERS

Five-hundredths per cent nordihydroguaiaretic acid, together with 0.01 per cent ascorbyl palmitate, offered much greater protection against peroxide accumulation and destruction of vitamin A than that obtained by using 0.05 per cent nordihydroguaiaretic acid alone or smaller concentrations of the antioxidant with and without 0.01 per cent ascorbyl palmitate.

NORDIHYDROGUAIARETIC acid, isolated from *Larrea divaricata* (1), has been shown to be a powerful antioxidant for animal fats (2), particularly those from swine and beef. Furthermore, the antioxidant effect is markedly enhanced by the presence of certain synergists, such as ascorbityl palmitate, citric acid, etc. It is not so marked in vegetable oils, although considerable protection is evidenced. In general, this is true with most antioxidants.

Fish liver oils are susceptible to oxidative rancidity and are accompanied by a considerable loss of vitamin A. This type of rancidity can be avoided in a large measure without the aid of antioxidants provided proper storage conditions are employed. However, in the case of cod-liver oil, opening of the original container and subsequent exposure to air is accompanied by an immediate oxidative reaction. Therefore, consumption of the oil within a reasonable length of time is imperative from both the standpoint of preventing the formation of an undesirable rancid oil and also the destruction of its vitamin A content.

EXPERIMENTAL

Studies involving the use of nordihydroguaiaretic acid as an antioxidant in cod-liver oil were initiated because of its marked antioxidant properties in connection with animal fats. These studies were conducted upon samples of cod-liver oil stored both at room and refrigerator temperatures. This was done because accelerated tests, i.e., involving higher temperatures with or without aeration, could not be interpreted in terms of days, weeks, months, etc. Nordihydroguaiaretic acid alone, in concentrations of 0.01 0.025, and 0.05%, and together with 0.01% ascorbyl palmitate were used. Controls were, of course, established. Ascorbyl palmitate was chosen as a type synergist because, in the concentrations used, it is readily soluble in cod-liver oil. Ten cubic centimeter samples were withdrawn once weekly to determine their peroxide contents according to the usual procedure (3). For want of an easily accessible and suitable photoelectric colorimeter, peroxide values in these preliminary studies were obtained only at definite intervals. However, the vitamin A content was determined at the end of approximately 26 weeks by the photoelectric colorimetric method using the conventional quantitative adaption of the Carr-Price antimony trichloride in chloroform color reaction with modifications described by Benham (4).

Nordihydroguaiaretic acid and ascorbyl palmitate were readily incorporated into the cod-liver oil by warming them with a small portion of the oil, cooling the same after solution had been effected and then diluting with fresh oil to the desired volume which, for these experiments, was 300 cc.

The results of the experimental work can be best expressed by graphs (Figs. 1 and 2).

DISCUSSION

The cod-liver oil used in these experiments was Parke, Davis & Co.'s high potency cod-liver oil that had an initial peroxide value of zero, and contained 2900 I. U. of vitamin A. The vitamin A content was determined colorimetrically as described above. Figure 1 represents samples stored at room temperature and the peroxide value plotted against weeks. The peroxide value is a measure of the amount of peroxide, due to oxidative rancidity, that has accumulated in the oil. It was calculated as follows (5).

$$\text{Peroxide number} = \frac{0.5\ (\text{ml. thiosulfate used in titration}) \times (\text{normality of thiosulfate}) \times 1000}{\text{wt. of fat}}$$

It will be noted that in the case of the 0.025 and 0.05 per cent, respectively, of nordihydroguaiaretic acid alone, the most significant and greatest protection is afforded up to the 20th and 18th weeks, respectively, after which time those that also contained 0.01% ascorbyl palmitate were much better protected. A sharp break in the peroxide value curves for the 0.025 and 0.05 % nordihydroguaiaretic acid occurred after the 20th week. Nordihydroguaiaretic acid 0.01 %, plus ascorbyl palmitate, 0.01 %, gave the least protection and, strange as it seems, was less stable for a period of time than the controls. On the other hand, 0.01 % nordihydroguaiaretic acid alone was slightly better than the control.

Facilities did not permit the determination of the vitamin A content of all the samples in a short

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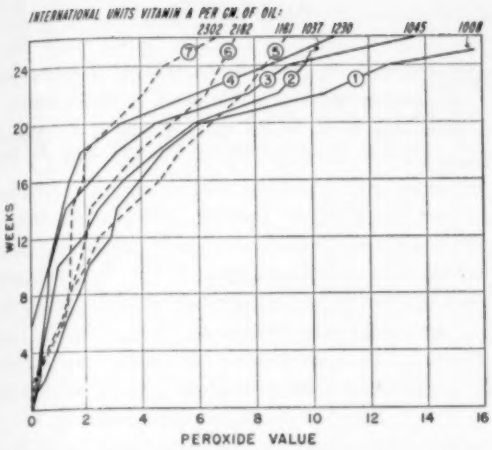

Fig. 1.—Preservative effect of varying concentrations of N. D. G. A. and of N. D. G. A. and ascorbyl palmitate on cod-liver oil stored at room temperature.

Legend: (*1*) Control (No N. D. G. A.); (*2*) 0.01% N. D. G. A.; (*3*) 0.025% N. D. G. A.; (*4*) 0.05% N. D. G. A.; (*5*) 0.01% N. D. G. A. + 0.01% ascorbyl palmitate; (*6*) 0.025% N. D. G. A. + 0.01% ascorbyl palmitate; (*7*) 0.05% N. D. G. A. + 0.01% ascorbyl palmitate.

period of time. Therefore, some samples were assayed for their vitamin A content 2 weeks later than others. If these samples had been assayed within several days, a closer correlation of the vitamin A content and time of storage would no doubt have been obtained. Nevertheless an over-all picture was gained in these preliminary studies which enabled conclusions to be drawn as to the efficacy of the antioxidant with and without the ascorbyl palmitate under the conditions of the experiment.

At about the 26th week, the samples containing 0.05 per cent nordihydroguaiaretic acid plus 0.01 per cent ascorbyl palmitate had a peroxide value of 7 and contained 2302 I. U. of vitamin A per Gm. of oil. Twenty-five thousandths per cent nordihydroguaiaretic acid, plus 0.01 per cent ascorbyl palmitate gave almost as good protection, i.e., peroxide value of 8.5 and contained 2182 I. U. of vitamin A per Gm. of oil. The control had the highest peroxide value, i.e., 15.5 and had the lowest vitamin A content, i.e., 1008 I. U. per Gm. of oil.

Figure 2 shows an almost immediate rise in peroxide value in all samples tested and after the 27th week all samples showed a much greater increase in their peroxide contents when compared to those samples stored at room temperature. However, the over-all relationships between the concentration of antioxidants to the controls when compared to the protective value of those stored at room temperature were somewhat similar. The greatest protection was evidenced in the sample containing 0.05 per cent nordihydroguaiaretic acid, plus 0.01% ascorbyl palmitate which, after the 27th week, had a peroxide value of 12.5 and contained 2445 I. U. of vitamin A per Gm. of oil. Nordihydroguaiaretic acid, 0.025 %, plus 0.01 per cent ascorbyl palmitate gave almost as good protection from the standpoint of the vitamin A content, i.e., 2230 I. U. per Gm. after the 27th week. However, the peroxide value rose to 16.5. The control had the highest peroxide content even after the 25th week, i.e., 29.5 and the lowest vitamin A content, i.e., 708 I. U. per Gm. of oil.

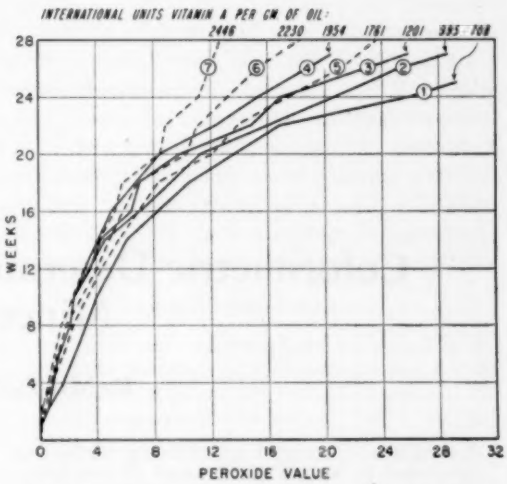

Fig. 2.—Preservative effect of varying concentrations of N. D. G. A. and of N. D. G. A. and ascorbyl palmitate on cod-liver oil stored at refrigerator temperature.

Legend: (*1*) Control (No N. D. G. A.); (*2*) 0.01% N. D. G. A.; (*3*) 0.025% N. D. G. A.; (*4*) 0.05% N. D. G. A.; (*5*) 0.01% N. D. G. A. + 0.01% ascorbyl palmitate; (*6*) 0.025% N. D. G. A. + 0.01% ascorbyl palmitate; (*7*) 0.05% N. D. G. A. + 0.01% ascorbyl palmitate.

CONCLUSIONS

(1) The peroxide value alone is not a measure of the amount of destruction of vitamin A, but must also be correlated with the temperature at which the peroxides were allowed to accumulate.

(2) The destruction of vitamin A in samples of cod-liver oil stored for 26 weeks at room temperature or in the refrigerator was essentially the same when 10-cc. portions were removed weekly and tested for their peroxide contents.

(3) Sample of cod-liver oil with or without nordihydroguaiaretic acid alone or with ascorbyl palmitate showed a greater peroxide concentration when stored in the refrigerator as compared to those stored at room temperature without a commensurate decrease in the vitamin A content after a period of about 26 to 27 weeks.

(4) Nordihydroguaiaretic acid, 0.05 per cent, together with 0.01 per cent ascorbyl palmitate,

offered much greater protection against peroxide accumulation and destruction of vitamin A than that obtained by using 0.05 per cent nordihydroguaiaretic acid alone and smaller concentrations of the antioxidant with and without ascorbyl palmitate.

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Colorimetric Determination of Peroxides in Mineral Oils*

By MILTON J. GOLDEN

A simple and accurate quantitative method is proposed by which the amount of peroxides formed in mineral oil is determined quantitatively by measuring in a Klett-Summerson photoelectric colorimeter the developing pink or red color of the oxidation of the ferrous ion to the ferric ion by the peroxides. Several mineral oil samples have been tested accordingly, and a table is given which compares the quantitative Peroxide Values of the oils with their respective Peroxide Tests. A graph is also given to facilitate the determination of the amount of peroxides in any 5 cc. of heated mineral oil. This quantitative method may be used for determining accurately the peroxide content of other oils, such as cod-liver oil or vegetable oil, and also for evaluating the effectiveness of antioxidants.

THE PRINCIPAL methods that are used today for estimating the peroxide content of such materials as cracked gasoline, hydrocarbons, oils, and fats are based upon the oxidation of either potassium iodide or a ferrous salt by the peroxides.

In 1929, Marks and Morell (1) proposed a method for determining the peroxides in oxidized linseed oil by titrating the free iodine, liberated from a solution of potassium iodide in glacial acetic acid, with a standard sodium thiosulfate solution. Two years later, Yule and Wilson (2) estimated the peroxides in cracked gasoline by shaking it with an acidified solution of ferrous sulfate and ammonium thiocyanate in an acetone-water mixture and determining the ferric thiocyanate formed with a standard titanous chloride solution.

Several modifications of these two basic methods have been suggested. Using the iodometric method for determining the peroxide content of fats and oils, Stuffins and Weatheral (3) investigated the sources of error in the method and modified it by using either chloroform or carbon tetrachloride for the extraction of the liberated iodine. Risbey and Nisbet (4) compared the various methods in respect to the peroxide value of "motor spirits" and modified the original Yule and Wilson method by using zinc to reduce the trivalent iron to the bivalent state. The peroxide content of unsaturated organic compounds (5) has also been estimated colorimetrically by determining the amount of ferric thiocyanate with color standards.

Various sources of error exist in these methods. In the iodometric method and its modifications, there may occur the reabsorption of the liberated iodine by the materials and the oxidation of the reagents by air. Yule and Wilson (2), after an exhaustive study of the oxidation of ferrous sulfate by cracked gasoline, observed that there still remained a small amount of peroxides which reacted with potassium iodide. Nevertheless, the ferrous salt method and its modifications have been found to be more suitable and useful for comparative purposes even though complete reduction of all the peroxides present is not obtained.

* Received Nov. 6, 1947, from the Research Laboratories McKesson and Robbins, Inc., Bridgeport, Conn.

Whereas most of the materials investigated have been either cracked gasoline or oils, the amount of peroxides formed in mineral oil upon standing has never been thoroughly discussed or studied. Thus far, two quantitative procedures have been suggested for the deterioration of mineral oil. Assaf and Gladding (6), using the Grignard reagent, methyl magnesium iodide, claim that they have been able to estimate the amount of peroxides in mineral oil by means of the complicated Grignard reaction apparatus of Larson. Rae (7), on the other hand, measured the degree of decomposition of various paraffin oils, heated to 150°C., by determining the volume of 0.1 *N* potassium permanganate reduced.

Both of these methods are impractical as routine examinations. The procedure recommended by Assaf and Gladding requires the use of a complicated apparatus and skilled workers. With Rae's method, only poorly refined paraffin oils can be properly studied and compared.

The purpose of this paper is to introduce a simple and accurate method, which can be used for the quantitative determination of the peroxides formed in mineral oil.

It is an established fact that mineral oil upon prolonged standing or exposure to sunlight will develop a peculiar kerosene-like odor and taste. This is due to the formation of peroxides in the oil. The development of these peroxides is reported to be less likely in more highly purified oils than in inferior types.

Several qualitative methods have been suggested for determining the stability of mineral oil. Samples of mineral oil have been exposed to sunlight and to ultraviolet light in filma-sealed, flint bottles (8) and then observed for the possible presence of odors and flavors. Mineral oil stability has also been studied spectroscopically (9), and its absorption bands compared. Some investigators have studied the stability of oxidized paraffin oils by measuring the change in interfacial-tension (10) between a layer of 0.02 *N* sodium hydroxide and the oil.

Whereas the previously mentioned tests for determining the stability of mineral oils have been based upon certain physical factors, stability can also be determined chemically by the oxidation of the ferrous ion to the ferric ion by the peroxides present in heat-treated oils. The developing pink to red color can then be measured colorimetrically.

Several oil companies and individual investigators (11) have used a colorless test solution of ferrous sulfate, ammonium sulfocyanate, and sulfuric acid for the detection of peroxides. The oils were heated either under steam pressure or by direct heat. The development of a pink or red color in the test solution indicated the presence of peroxides, which constituted an objection with regard to stability.

A Peroxide Test method (12) has recently been proposed in which the expected shelf-life or stability of mineral oil is expressed in time units. The method consists of adding a colorless test solution of ferrous sulfate, ammonium thiocyanate, sulfuric acid, reduced iron, and iron wire to samples of oil heated in a 300° F. oil bath for various intervals and observing the developing color. The color is reported to vary from colorless to dark red, depending upon the amount of peroxides formed during the breakdown of the oil. The first sample exhibiting a pink coloration after shaking with the test solution is regarded as the Peroxide Test value, expressed in minutes.

EXPERIMENTAL

These facts and reactions have been used in developing the following quantitative method for the determination of peroxides formed in mineral oil. Samples of mineral oil were left in a 300° F. oil bath for one hour, and a colorless test solution, consisting of ferrous sulfate, ammonium thiocyanate, sulfuric acid, and reduced iron, was added to the heated oil. The developing pink or red color of the test solution was then measured colorimetrically in a Klett-Summerson photoelectric colorimeter.

Apparatus.—One-liter round-bottom flask and reflux condenser; test tubes $^3/_4 \times 6$ inches; constant temperature oil bath regulated at 300° F. ± 1° F.; glass-stoppered reaction flasks with greaseless stop cock, according to Connor and Straub's vitamin B_1 fluorometric method; centrifuge; Klett-Summerson photoelectric colorimeter with filter No. 56, having a mean transmission of 560 mμ, and special test-tube cells.

Materials.—Ferrous Sulfate C. P.; Ammonium Thiocyanate, C. P.; Iron by Hydrogen, Merck (reduced iron); Iron Wire No. 30, for standardization; Acetone U. S. P.; 3% Hydrogen Peroxide Solution.

Preparation of Test Solution.—In a round-bottom, wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc. concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder 0.2 Gm. and 6 inches of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction process takes about two hours, it being necessary to continue refluxing to insure lack of color.

Fresh solution is prepared on the day of the test. It is possible, however, to preserve it in a colorless state by careful storage in a carbon dioxide atmosphere.

Since it is necessary to avoid any contact with air, a dispensing method has been devised. A syphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole

is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the syphon, at the end of which is attached a glass stopcock for the withdrawal of the solution.

Procedure.—Samples of mineral oil to be tested are placed in individual test tubes and immersed in a constant temperature oil bath at 300° F. ± 1° F. The tubes are removed after one hour's immersion in the oil bath and allowed to cool to room temperature. A 5-cc. sample of the heated mineral oil is pipetted into a glass-stoppered reaction flask which has been marked to indicate 5 cc. and 10 cc. filling. The air in the flask is replaced with CO_2, and 5 cc. of the colorless test solution is added. The flask is then tightly stoppered with the glass stopper, shaken by hand for one minute, and centrifuged for three minutes. The lower test solution layer which will then vary in color from pink to dark red is transferred quantitatively by means of the greaseless stopcock from the reaction flask to a cork-stoppered special Klett test tube cell, in which the air has also been replaced with CO_2. As a control, 5 cc. of unheated oil is treated similarly and run parallel with the heated sample. The test-tube cells, containing the sample and control, are then placed as soon as possible in the Klett colorimeter, and the amount of pink or red color formed is measured using the Klett filter No. 56. The difference in the scale readings between the unheated and heated oil samples gives the reading for the oxidation of ferrous thiocyanate in the test solution by the peroxides in 5 cc. of heated oil.

Prior to the above-described procedure, the Klett photoelectric colorimeter is adjusted until a zero reading is obtained with the colorless test solution and then standardized with various concentrations of hydrogen peroxide by measuring the amount of color corresponding to various quantities of peroxide. A freshly prepared 3% hydrogen peroxide solution is diluted 1–100, and its peroxide content is determined according to the U. S. P. assay method with 0.01 *N* potassium permanganate. Extremely small volumes of this diluted peroxide solution, having a concentration range of 1×10^{-6} to 1×10^{-4}, are added to 5-cc. portions of the colorless test solution in cork-stoppered Klett test-tube cells, and the amount of pink or red color formed is measured in the colorimeter.

Hydrogen peroxide concentrations are plotted against scale readings, and Fig. 1 represents the standardization curve obtained with increasing concentrations of peroxide. This graph facilitates the determination of the amount of peroxides in any 5-cc. sample of heated mineral oil by evaluating the concentration corresponding to the difference in scale readings between the control and sample.

It is essential that this standardization of the colorimeter be repeated on the day on which the test is to be carried out to correct any deviation in the colorimeter and possible changes in the standard peroxide solution.

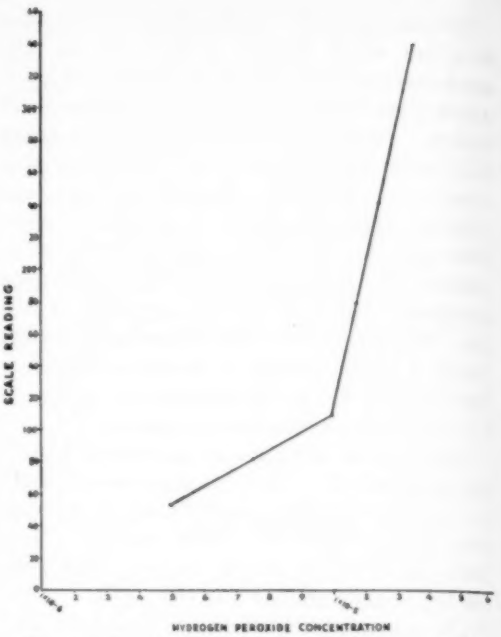

Fig. 1.—Standardization curve showing relationship of scale readings to hydrogen peroxide concentrations.

DISCUSSION

The described procedure makes possible not only the evaluation of the peroxides in mineral oil, but also the comparison of oils which, with the known methods of testing, would appear to have similar stability and quality.

Samples of mineral oil of several brands were tested accordingly. Table I summarizes the comparison of the Peroxide Values of the oils with their Peroxide Tests. The Peroxide Value is a quantitative expression for the amount of hydrogen peroxide formed in one liter of mineral oil, heated for one hour at 300° F. The Peroxide Test, on the other hand, is a qualitative test in which minutes of heating and detection of the degree of peroxides formed provide a means for establishing the stability of a mineral oil for various lengths of time.

TABLE I.—COMPARISON OF PEROXIDE VALUES WITH PEROXIDE TESTS OF MINERAL OILS

Sample of Various Brands	Peroxide Values, Grams of Hydrogen Peroxide per Liter of Heated Oil	Peroxide Test, Min.
A	0.00086	35
B	0.00116	30
C	0.00122	30
D	0.0012	22
E	0.005	15
F	0.0098	15
G	0.009	12
H	0.0116	12
I	0.0118	10
J	0.0182	10

The Peroxide Value reported for each sample represents the average of at least three separate determinations, which never varied more than ±3%.

The results obtained confirm and substantiate what has already been reported, namely, that an oil which develops peroxides after two minutes' heating is considered unstable and of poor shelf-life. Moreover, an oil which gives a colorless test with the ferrous thiocyanate solution after being heated twenty minutes or longer can be regarded stable and of very good quality (12).

The accuracy and reproducibility of the colorimetric method presented have been studied by means of dilute solutions of hydrogen peroxide of known concentration and by means of pure preparations of succinyl peroxide. For example, the concentration of hydrogen peroxide solution as given by the standard potassium permanganate method was 0.0036 mole per liter, while the colorimetric procedure gave 0.0037 mole per liter. The succinyl peroxide was prepared by the method of Clover and Houghton (13) and was recrystallized twice from acetone. The calculated weight per cent of active oxygen in succinyl peroxide is 6.83%; obtained by the colorimetric procedure 6.9%.

This suggested peroxide method offers a means of determining a new constant or property for both medicinal mineral oils and oils used in pharmaceutical and cosmetic preparations. Measurement of the amount of peroxides and the expected stability of different oils is made possible.

In the pharmaceutical use of mineral oil, it is of the utmost importance that careful consideration be given to the factor of stability. The values obtained by the above-described procedure offer themselves as a yardstick in this respect. Oils having a hydrogen peroxide content less than 0.005 gram per liter after heating for one hour at 300° F. will be of a high purity and stability and, therefore, suitable for prolonged medicinal use.

This quantitative method may also be used for determining accurately the amount of peroxides in other types of oil and may facilitate the evaluation of the effectiveness of antioxidants in those oils and preparations that readily turn rancid.

SUMMARY

A simple and accurate quantitative method is proposed for the determination of peroxides formed in mineral oil.

This colorimetric method may be used for estimating the peroxide content of other types of oil, such as cod-liver oil or vegetable oil, and also for evaluating the effectiveness of antioxidants.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

N-Methylhydroxylamine
Galactoflavin
2-Hydroxyundecylenic acid
2-Hydroxycaprylic acid
Avidin
Dihydrodypnone
Auxin A and B
Methionic acid
Coumarin-3-carboxylic acid
Urobilin
Dihydro-γ-benzopyrone
8-Methoxydihydro-γ-benzopyrone
Fluoromalonic acid
Chloromalonic acid
Dibenzofurane-3-sulfonic acid
2-(m-Hydroxyphenyl)ethylamine
N-Methyldiiodochelidamic acid
Thiophosgene

Magnesium Stearate. I. A Comparative Study of Commercial Samples*

By MARION L. LIEN† and C. E. MILLER‡

A comparative study of ten commercial samples of magnesium stearate involving physical and chemical tests has been made. The variable results obtained indicate that the samples were not identical in composition.

INCREASED PRODUCTION of commercial grades of magnesium stearate has been noted during the past five years. A survey of the literature disclosed the fact that no published comparative study of commercial grades of this chemical has been made. The importance of magnesium stearate to the cosmetic and paint industries, together with the lack of data concerning the relative compositions of available grades, made it of definite interest to conduct a comparative study. It is the purpose of this paper to present data obtained from such a study. Ten commercial samples were obtained from industry for this investigation and were numbered from one to ten inclusive.

Experimental

Analysis of Magnesium Stearate.—The several samples were characterized by studying their physical appearance, softening point, melting point, moisture, solubility in acetone and benzene, total ash, and magnesium content. The results of these determinations are summarized in the following tables.

TABLE I.—CHEMICAL AND PHYSICAL COMPARISON OF MAGNESIUM STEARATE

Sample	Softening Point, °C.	Melting Point °C.	Moisture, %[a]	Total Ash as MgO, %[b]
1	123	172–179	3.49	10.63
2	104	138–145	5.13	8.24
3	126	170–178	3.25	8.37
4	127	169–178	3.13	8.75
5	112	145–151	3.53	8.99
6	128	169–178	2.88	8.06
7	115	139–142	2.49	7.90
8	121	169–177	3.22	10.04
9	111	144–150	4.37	8.77
10	132	168–174	3.17	9.20

[a] Covered dishes used during weighing.
[b] Reported on a moisture-free basis.

* Received Jan. 5, 1948, from the School of Pharmacy, North Dakota Agricultural College, Fargo, N. D.

† Abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Chemistry, by Marion L. Lien, American Foundation for Pharmaceutical Education Fellow.

‡ Professor of Pharmaceutical Chemistry, School of Pharmacy, North Dakota Agricultural College.

It is evident from Table I that the magnesium stearate samples were not of identical composition. In the determination of the melting point it was noted in each case that the column of sample in the melting-point tube appeared to shrink or soften well below the melting-point range but not uniformly for the various samples. This phenomenon is reported under the heading of softening point. The melting-point bath was cooled to approximately 50° after each determination before again being used.

The theoretical amount of MgO that can be formed by the thermal decomposition of magnesium stearate, 6.82%, is less than the observed values, which is indicative of unreacted MgO or $Mg(OH)_2$. All of the samples were white in color, unctuous to the touch, and in a very fine state of particle size. Although pure magnesium stearate is reported to be soluble in acetone and in benzene (1), the samples studied were found to be incompletely soluble in these solvents, which is considered further proof that unreacted MgO or $Mg(OH)_2$ is present.

The pyrophosphate method used was essentially that of Kolthoff and Sandell (2), modified to make it applicable to magnesium stearate. Approximately 1.5-Gm. samples were ignited in nickel crucibles to constant weight and the resulting ash dissolved in 1:1 HCl. The magnesium was then precipitated in the usual manner as magnesium ammonium phosphate, collected on tared Gooch crucibles, washed free of chlorides and ignited to magnesium pyrophosphate. The above results are the average of duplicate determinations, expressed on a moisture-free basis.

Goldstein (3) determined magnesium by precipitating a solution of the magnesium salt with 8-hydroxyquinoline, thereby obtaining a yellow precipitate of magnesium hydroxyquinolate. This method, somewhat modified by Martin and Green (4), is the basis of assay for magnesium in Magnesium Citrate Solution. Application of this method to magnesium stearate samples was accomplished by liberating the magnesium from the soaps by acid hydrolysis. The resulting filtrates were made up to volume and analyzed. Twenty-five-cc. aliquots of the filtrate were precipitated as the 8-hydroxyquinolate, filtered, and dried to constant weight. The above results are the average of duplicate determinations, expressed on a moisture-free basis.

Analysis of Liberated Fatty Acids.—Because of the variances shown by the several samples in the analysis of magnesium stearate, it was believed that a study of the acidic portion of the soap would yield interesting and valuable information. Accordingly one hundred-gram quantities of the soaps were hydrolyzed with dilute sulfuric acid, and after purification the liberated fatty acids were evaluated by appropriate methods. The results of this part of the investigation are given in Table III.

The physical appearance of the liberated fatty acids both at room temperature and in the liquid

TABLE II.—PER CENT MAGNESIUM IN MAGNESIUM STEARATE

Sample	Mg, % Method A[a]	Mg, % Method B[b]	Sample	Mg, % Method A[a]	Mg, % Method B[b]
1	4.88	4.40	6	5.17	4.44
2	4.68	4.25	7	4.84	4.03
3	4.72	4.53	8	4.94	4.64
4	4.50	4.00	9	4.77	3.99
5	4.62	4.43	10	4.66	4.60

[a] Method A—pyrophosphate method.
[b] Method B—8-hydroxyquinolate method.

TABLE III.—COMPARATIVE ANALYSIS OF LIBERATED FATTY ACIDS

Sample	M. P., °C.	Titer, °C.	$n^{71.8}_{D}$	Iodine Value	Acid Value	Sp. Gr. 97° C.	Mol. Wt.
1	64.0	57.0	1.4348	1.25	180.6	0.8636	386
2	62.5	58.5	1.4331	4.00	196.7	0.8638	340
3	67.5	58.0	1.4351	1.76	170.9	0.8649	426
4	66.5	60.0	1.4364	3.23	167.2	0.8681	359
5	64.0	56.5	1.4332	3.21	189.9	0.8619	449
6	66.5	56.0	1.4358	2.43	166.8	0.8677	420
7	57.0	55.0	1.4324	3.70	198.4	0.8641	368
8	68.5	63.5	1.4352	2.44	167.5	0.8661	455
9	65.0	61.0	1.4328	4.59	196.4	9.8627	375
10	66.0	59.5	1.4352	2.63	158.1	0.8725	472

state was comparable to Eastman Kodak Company, white label Stearic Acid. The melting points reported were determined by the class II method of the U. S. P. The refractive index was determined in the usual manner, a circulating constant temperature bath set at 75° was satisfactory to maintain the reported temperature. The Rast Camphor Method (5) was used for determining the molecular weights. A future paper will discuss the distillation analysis of the several commercial samples.

SUMMARY AND CONCLUSIONS

1. Ten commercial samples of magnesium stearate have been compared by physical and chemical means.

2. Variations observed in melting points, solubility, total ash, and moisture content indicated that the samples were not of uniform composition.

3. Variable results by both methods for the determination of magnesium are in accord with the observations that commercial samples of magnesium stearate are mixtures.

4. The 8-hydroxyquinolate method for determining magnesium is applicable with certain modifications to the analysis of commercial samples of magnesium stearate.

5. The somewhat lower values found by the 8-hydroxyquinolate method may be due to solubility of the magnesium hydroxyquinolate in the precipitating media.

6. Further evidence of the variable composition of the samples was demonstrated in the analytical results observed in evaluating the liberated fatty acids.

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Estimation of Some Official Salts of Weak Organic Acids by Titration in a Nonaqueous Medium*

By MELVIN W. GREEN

A study was made of the potentiometric titration of a number of official salts of weak organic acids, notably sodium benzoate, sodium acetate, sodium propionate, barbital sodium, phenobarbital sodium, sulfathiazole sodium and sulfadiazine sodium, using a solvent mixture of propylene glycol and isopropyl alcohol and perchloric acid in the same solvent as a titrant. The results were compared with results obtained by the empirical use of indicators. In some instances when the potentiometric end point, determined with a Fisher titrimeter, gave low results, the empirical use of methyl orange or methyl red gave satisfactory results.

MOST ACIDIMETRIC and alkalimetric methods of analysis are based upon the older classical laws of the dissociation of acids and bases. Since most of the reactions take place in aqueous solutions or solutions predominantly aqueous there is no reason for not accepting the classical theories as they explain all of the phenomena taking place which need explanation.

In 1923, Bronsted and Lowry, working independently, developed a theory that acids should be defined as substances having a tendency to lose a proton (H^+) and bases as substances having a tendency to gain a proton. Since it is unlikely that protons ever exist free in solution, the acidic or basic properties are never seen independent of the solvent and indeed the solvent itself must exist as a proton donor or acceptor in order for dissociation to take place, e.g., a solution of an acid in petroleum benzin shows no conductivity.

If the protogenic or protophilic property of the solvent plays a role in the dissociation of an acid or base, it then follows that a substance may have its properties materially altered by a change in solvent. Such is indeed the case, and to cite a somewhat extreme case, nitric acid actually behaves as a base when dissolved in strong hydrogen fluoride solution where the following equilibrium is set up.

$$\underset{\text{base}}{HA} + \underset{\substack{\text{acid}\\(\text{solvent})}}{HF} \rightleftarrows \underset{\text{acid}}{H_2A^+} + \underset{\text{base}}{F^-}$$

The weaker an acid in aqueous solution the more likely it is to be a proton acceptor; hence dissolved in a protogenic solvent the more apt it is to behave like a strong base. The strength of bases is also influenced by the acidic or basic character of the solvent. An amine will thus behave as a very weak base in ammonia, a moderate base in water and as a very strong base in acetic acid (1, 2).

These facts have led several workers to carry on titrations in media other than water. Work has been somewhat slow from a practical viewpoint because the scale of acidity still has not been thermodynamically established and the value for the liquid junction potential is unknown in nonaqueous solutions. This brings about the very practical point of indicator selection for one does not know either the true *p*H of the end point of an acid-base titration, or the *p*H range of indicators under these conditions.

A further real handicap has been the limited solubility of many substances to be titrated. For example, although the sodium salts of most weak acids behave as strong bases when dissolved in alcohol, many of these salts are only moderately alcohol-soluble. Sodium benzoate, for example, is soluble 1 in 2, in water, but only 1 in 75, in alcohol; sodium propionate is soluble 1 in 1, in water, but only 1 in 24, in alcohol. Some of the other solvents are even poorer in this respect.

In 1946, Palit (3) showed that advantage could be taken of the solvating properties of the glycols on such salts, the $R.COO^-$ ion reacting with the glycol by hydrogen bonding to form a complete solution. If R is large enough, however, solution cannot be effected without the addition of a better hydrocarbon solvent such as isopropyl alcohol or butyl alcohol. Furthermore, it is difficult to titrate in a pure glycol medium because of the high viscosity of the glycol. By the use of 0.1 *N* to 0.2 *N* perchloric acid in a mixture of equal parts of propylene glycol or ethylene glycol and isopropyl alcohol, Palit was able to titrate such salts as sodium acetate and sodium propionate using either an indicator such as methyl orange (the choice of indicator is empiric) or a potentiometric end point in terms of apparent *p*H.

We set out to try to duplicate Palit's work and

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to explore the possibility of titrating such compounds as the sodium barbiturates and the sodium sulfonamides which are not carboxylic salts.

EXPERIMENTAL

The 0.1 *N* to 0.2 *N* perchloric acid was prepared by mixing approximately the correct quantity of 70% perchloric acid with a sufficient volume of equal parts propylene glycol and isopropyl alcohol. The small quantity of water in the 70% perchloric acid was the only amount of water in the entire system. This acid was standardized against 0.1 *N* alcoholic potassium hydroxide which in turn had been freshly standardized against pure potassium acid phthalate using methyl red as the indicator.

The salt or sodium compound was "dissolved" in a mixture of equal parts by volume of propylene glycol and isopropyl alcohol. At times solution was not complete but when the acid liberated (acetic, propionic, barbital, etc.) was sufficiently soluble in the mixed solvent to render solution complete when a fourth to a half of the reaction was completed, the method was considered to be satisfactory.

In nearly all cases the solution was stirred by a mechanical stirrer during the titration. Adequate agitation is more important in such titrations than in simple aqueous solutions for the viscosity of the solutions tends to make the reaction sluggish. Of equal importance is to wait at least one minute after adding titrant from the burette to obviate drainage error due to the viscosity of the solution.

In the potentiometric titrations, a Beckman *p*H meter with the conventional calomel and glass electrodes was used in the earlier studies. Later a Fisher titrimeter which employs the same electrode system, but has the additional advantage of a cathode tube (magic eye) indicator, was used. In the former case hand stirring was used.

In the indicator method, methyl orange, methyl red and, in a few instances, thymol blue, were used. It is worthy of note that these indicators gave much brighter colors in the mixed solvent but methyl orange still gave some of the traditional difficulty in ascertaining the exact end point. In almost every instance the indicator method used more titrant than the potentiometric method, i.e., the indicator gave a false end point beyond the true potentiometric end point. In some instances, as for example barbital sodium, this error actually enables one to obtain substantially the correct value for the analysis.

In Table I it may be seen that fairly good results were obtained in most of the salts determined, but that there is some tendency for the results to be high. Sodium propionate gave somewhat low results potentiometrically, however, as did sodium benzoate when the titrimeter was used. The discrepancy between the results for sodium benzoate using the *p*H meter and those using the titrimeter is difficult to explain unless with hand stirring the salt was overtitrated.

In Table II it may be seen that pentobarbital sodium gave equally low results with all three methods and barbital sodium gave good results when a colorimetric end point was chosen but poor results on either potentiometer.

From Table III it may be seen that sulfadiazine sodium gave very erratic results under all conditions. This can probably be attributed to the relative insolubility of both the sodium salt and the acid form of the drug in the medium employed. Sulfathiazole sodium, on the other hand, reacted very favorably.

The author considers this paper to be preliminary, for while it was shown that the method has possibilities it is believed that there are certain factors such as modification of solvent, speed of titrating, and indicator choice which need further study.

TABLE I.—NONAQUEOUS TITRATION OF SODIUM SALTS OF WEAK ACIDS

Salt	End Point	Recovery by Nonaqueous Titration %	Official Method, %
Sodium Acetate, N. F.	Potentiometric	100.00	99.93
		100.24	99.87
Sodium Propionate, N. F.	Potentiometric	98.2	99.5
		98.8	99.2
	Colorimetric	99.1	99.3
Sodium Benzoate, U. S. P.	Potentiometric	99.73[a]	98.95
			99.24
		97.27[b]	99.18
	Colorimetric	100.6	
		99.64	
Sodium Salicylate, U. S. P.	Potentiometric	100.23	99.52
	Colorimetric	100.23	99.60

[a] *p*H meter.
[b] Fisher titrimeter.

SUMMARY

1. Using 0.1 *N* to 0.2 *N* perchloric acid in a mixture of equal parts of propylene glycol and isopropyl alcohol certain sodium compounds have

TABLE II.—NONAQUEOUS TITRATION OF SODIUM BARBITURATES

Compound	End Point	Recovery by Nonaqueous Titration, %	Recovery by Official Method, %
Barbital Sodium, U. S. P.	Potentiometric	95.66[a]	
		96.40[b]	
			99.32
			99.15
	Colorimetric	99.1	
		99.1	
Phenobarbital Sodium, U. S. P.	Potentiometric	100.2[a]	
		99.15[b]	
			98.93
			99.24
	Colorimetric	100.7	
		100.3	
Pentobarbital Sodium, U. S. P.	Potentiometric	97.57[a]	
		96.67[b]	
			99.27
			99.46
	Colorimetric	97.62	
		97.48	

[a] pH meter.
[b] Fisher titrimeter.

TABLE III.—NONAQUEOUS TITRATION OF SODIUM SULFONAMIDES

Compound	End Point	Recovery by Nonaqueous Titration, %	Recovery by Official Method, %
Sulfadiazine Sodium, U. S. P.	Potentiometric	101.5[a]	
		93.4[b]	
		88.8[b]	
			99.68
			99.73
	Colorimetric	101.6	
		103.1	
		100.1	
Sulfathiazole Sodium, U. S. P.	Potentiometric	99.33[a]	
		100.9[a]	
		99.3[b]	
			99.08
			99.14
	Colorimetric	99.42	
		99.52	

[a] pH meter.
[b] Fisher titrimeter.

been titrated using indicator dyes and potentiometers to determine the end point.

2. Sodium acetate, sodium benzoate, sodium salicylate, phenobarbital sodium, and sulfathiazole sodium gave favorable results. Barbital sodium gave low results with a potentiometric titration but good results when an indicator was used. Pentobarbital sodium gave relatively low results by all methods. Sulfadiazine sodium cannot be titrated in this manner, at least without considerable modification of the method.

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An Improved Procedure for the Extraction of Phlorizin*

By GEORGE D. CURTS†

A method is given whereby phlorizin is obtained, from fresh apple root bark, as a pearly-white, lustrous solid.

PHLORIZIN is a glucoside found in the root bark of various species of apples in the amount of about 0.2%. It is known to lower the threshold of sugar retention by the kidneys, thus inducing a glycosuria. Continued interest (1) in the pharmacological properties of phlorizin has encouraged the search for improvements in the extraction procedure.

EXPERIMENTAL

The fresh root bark is collected and shredded in a hammer mill very soon thereafter, although it may be stored in a refrigerated room for a day or so if necessary. The bark shreds very well at a 1/4-inch mesh setting. After shredding, the bark is immersed in boiling water and steeped for one hour in a standard manner (2), steam being introduced to maintain the boiling temperature. The resulting mash is strained and the steeping process repeated. The material is again strained and the combined strained liquids allowed to stand for twenty-four hours. With some species, impure phlorizin will separate from the menstruum at this time.

If phlorizin does not separate, the solution is evaporated to dryness under reduced pressure, or, if preferred, under normal pressure with the addition of calcium or magnesium carbonate when nearly dry. The residue is then extracted with hot ethyl acetate and this solution concentrated.

Impure phlorizin may be precipitated by the addition of chloroform, but interfering resinous material is also precipitated. This material may be removed by first precipitating with two volumes of ether. The phlorizin remains in solution and is obtained by the addition of one volume of chloroform.

The phlorizin precipitate is then purified by treatment with activated charcoal in hot aqueous solution, or by alternate aqueous and ethyl acetate-chloroform crystallization.

Phlorizin obtained by this method is a pearly-white, lustrous solid. The hydrous form melts at 114° (unc.) and the anhydrous at 158° (unc.)

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† Graduate student, Ohio State University.

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Diethylaminoethyl Dialkylacetates*

By RAMAN C. AMIN, WALTER H. HARTUNG, and CLIFFORD W. CHAPMAN

The synthesis is reported of a series of diethylaminoethyl esters of dialkylacetic acids in which the carbon content varied from six to eight atoms. Four esters of alpha-bromo acids were also prepared. All compounds described were tested for surface anesthetic action but none was effective.

PROCAINE analogs in which the acyl component of the molecule is aliphatic are not new. Diethylaminoethyl acetate shows no anesthetic action (1). The corresponding esters of the higher aliphatic, straight-chain acids exhibit some activity; their effectiveness is reported to be proportional to their molecular weight (2, 3). Unsaturation in the straight carbon chain of the acid apparently modifies the pharmacological properties but little (2), although the esters of acrylic acid derivatives, $RCH{=}CHCOOCH_2CH_2N(C_2H_5)_2$ are distinctly, though weakly, active (4).

Among these procaine analogs little or no mention is made of the esters derived from

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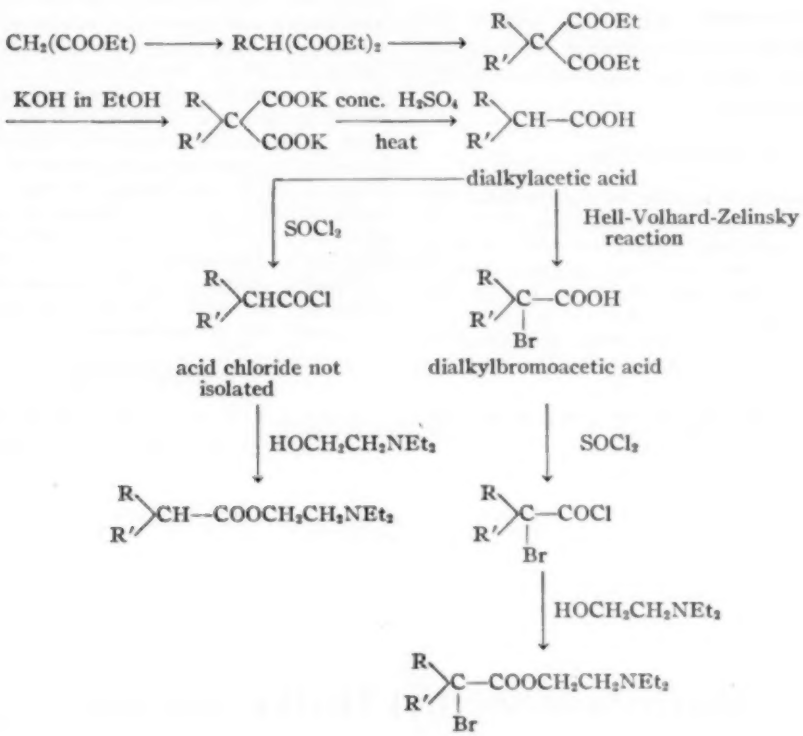
branched-chain aliphatic acids. In view of the fact that branched-chain compounds frequently differ in pharmacological properties, either quantitatively or qualitatively, from their normal chain isomers, it was felt that the dialkylaminoalkanol esters of branched-chain acids merited investigation. Accordingly a series of diethylaminoethyl esters of dialkylacetic acids were prepared in which the carbon content varied from six to eight atoms. Since the presence of halogen in an organic molecule increases lipoid solubility, four esters of α-bromo acids were also prepared.

EXPERIMENTAL

The compounds were prepared according to the sequence of reactions shown below:



The acids obtained are listed with pertinent data in Table I.

The acids were allowed to react with thionyl chloride. The resulting acid chloride was not isolated, but was dissolved in dry benzene, treated directly with a solution of diethylaminoethanol in benzene, and the mixture refluxed for one hour. The organic mixture was washed with aqueous potassium hydroxide solution and the organic layer was then dried over anhydrous sodium sulfate before distillation. The desired ester was collected at the proper point. A typical synthesis, that of diethylaminoethyl *n*-butylethylacetate, is described below.

Diethylaminoethyl *n*-Butylethylacetate.—In a 500-cc., 2-neck, round-bottom flask, equipped with a dropping funnel and reflux condenser which was connected to an absorption trap for hydrogen chloride, was placed 21.6 Gm. (0.15 mole) 2-ethylhexanoic acid (*n*-butylethylacetic acid). This was heated on a water bath, and during the course of a half hour 24 Gm. (0.2 mole) of thionyl chloride was added through the dropping funnel. The mixture was heated for about three hours until all the acid was dissolved; excess of thionyl chloride was removed by distillation. The acid chloride was dissolved in 40 cc. dry benzene. The two dropping funnels containing this solution and 19.92 Gm. (0.17 mole)

In order to evaluate the local anesthetic activity of these esters, 2 per cent solutions of the compounds in sterilized water having the *p*H adjusted between 6 and 7 were tested by the rabbit's cornea method described by Schmitz (5) and Harry (6) and their co-workers. An unbuffered 1 per cent aqueous solution of cocaine was used as the control.

None of the esters prepared showed any anesthetic activity in 2 per cent solution; when the concentration was increased to 6 per cent there was still no apparent anesthesia, but severe irritation and inflammation were produced.

TABLE I.—DIALKYLACETIC ACIDS

RR'CHCOOH R	R'	B. P. in °C. at 18 Mm.	Density d_{23}^{23}	Formula	Neutralization Equivalent Calcd., %	Found, %
Ethyl[7]	Ethyl	99–101	0.9237	$C_6H_{12}O_2$	116.13	117.42
n-Propyl[8]	Ethyl	105–107	0.9361	$C_7H_{14}O_2$	130.15	131.43
Isopropyl[9]	Ethyl	110–112	0.9076	$C_7H_{14}O_2$	130.15	132.15
n-Butyl[10]	Ethyl	118–120	0.9029	$C_8H_{16}O_2$	144.17	141.66
Isobutyl[11]	Ethyl	120–122	0.9023	$C_8H_{16}O_2$	144.47	143.15
RR'CBr—COOH R	R'	Yield on Bromination, %				
n-Butyl[12]	H	51.76	136–138	1.2812	$C_6H_{11}O_2Br$	195.04	191.92
Isopropyl	Ethyl	33.4	123–125	1.0680	$C_7H_{13}O_2Br$	209.06	211.94
n-Butyl	Ethyl	31.2	145–148	1.2900	$C_8H_{15}O_2Br$	223.08	221.41
Isobutyl	Ethyl	48.1	127–129	1.0313	$C_8H_{15}O_2Br$	223.08	226.35

TABLE II.—DIETHYLAMINOETHYL DIALKYLACETATES

RR'CH—COOCH$_2$-CH$_2$N(C$_2$H$_5$)$_2$ R	R'	B. P. in °C. at 18 Mm.	Yield, %	d_{22}^{22}	n_D^{31}	Formula	Nitrogen Analysis (Kjeldahl) Calcd., %	Found, %
n-Propyl[3]	H	103–105	53.4	0.8862	1.4299	$C_{10}H_{21}O_2N$	7.48	7.62; 7.64
n-Butyl[3]	H	131–133	63.6	0.8937	1.4329	$C_{12}H_{25}O_2N$	6.51	6.73; 6.71
Ethyl	Ethyl	109–111	53.6	0.8858	1.4294	$C_{12}H_{25}O_2N$	6.51	6.43; 6.42
n-Propyl[3]	Ethyl	115–118	46.5	0.8946	1.4347	$C_{13}H_{27}O_2N$	6.11	5.87; 5.88
Isopropyl	Ethyl	120–122	43.6	0.8938	1.4339	$C_{13}H_{27}O_2N$	6.11	6.34; 6.32
n-Butyl[3]	Ethyl	127–129	66.6	0.8965	1.4366	$C_{14}H_{29}O_2N$	5.76	5.91; 5.89
Isobutyl	Ethyl	133–135	43.2	0.8960	1.4360	$C_{14}H_{29}O_2N$	5.76	5.61; 5.59
RR'CBr—COOCH$_2$CH$_2$N(C$_2$H$_5$)$_2$ R	R'							
n-Butyl	H	119–121	25.0	...	1.4442	$C_{12}H_{24}BrO_2N$	4.76	4.61; 4.58
Isopropyl	Ethyl	127–129	9.7	...	1.4478	$C_{13}H_{27}BrO_2N$	4.54	4.41; 4.44
n-Butyl	Ethyl	122–124	9.3	...	1.4476	$C_{14}H_{29}BrO_2N$	4.34	4.27; 4.29
Isobutyl	Ethyl	135–137	12.9	...	1.4461	$C_{14}H_{29}BrO_2N$	4.34	4.40; 4.41

of diethylaminoethanol in 40 cc. benzene, respectively, were fitted to a 3-neck round-bottom flask equipped with a mechanical stirrer. The two solutions were added dropwise and simultaneously with stirring to 40 cc. of benzene in the flask. The mixture was refluxed for one hour, then poured into 200 cc. of water, and made alkaline with 10% KOH solution. The benzene layer was separated and dried over anhydrous sodium sulfate and the benzene was removed by distillation under atmospheric pressure. The residue was distilled under reduced pressure to obtain a yield of 23 Gm. (66.6% of the theoretical amount) of diethylaminoethyl 2-ethylhexanoate, b. p. 127–129° 18 mm.

SUMMARY

(1) Nine branched-chain aliphatic acids were prepared, of which α-bromo-iso-propylethylacetic acid, α-bromo-*n*-butylethylacetic acid, and α-bromo-iso-butylethylacetic acid are not described in the available literature.

(2) Seven diethylaminoethyl dialkylacetates and four diethylaminoethyl α-bromodialkylacetates were prepared; of these the hydrochlorides of diethylaminoethyl *n*-butyrate, caproate, and heptoate; and diethylaminoethyl *n*-octoate have been previously described.

(3) The results of the pharmacological studies of these esters indicate that none possesses any surface anesthetic activity.

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Detoxification of Pyrogens by Hydrogen Peroxide in Some U. S. P. Injections*

By ABRAHAM TAUB† and FANCHON HART‡,§

The detoxification of pyrogens by boiling with a low concentration of hydrogen peroxide has been found applicable to water for injection, normal saline solution, and dextrose and sodium chloride injection. Excess peroxide is decomposed with manganese dioxide, or in the case of dextrose-saline, by activated charcoal. Selas candle filtration removes the decomposing agent. The method produces no significant alteration in the composition of the injection fluids.

THE REMOVAL of bacterial pyrogens from parenteral solutions has been the subject of considerable attention since Hort and Penfold (1) first showed that these fever-producing substances could not be eliminated by conventional sterilization techniques. Adsorption filtration through specially prepared asbestos pads has been the most widely accepted procedure for pyrogen removal from solutions of crystalloidal substances. That this method has not proved universally acceptable is indicated by the modifications which have been recommended. Francke and Reese (2) have advocated charcoal adsorption and filtration, followed by Seitz pad filtration. Simpler adsorption techniques using activated charcoal have been utilized for the removal of limited amounts of pyrogen from solutions containing nonadsorbable active ingredients (3). More recently, Smith and Pennell (4) have suggested the removal of pyrogen from plasma protein solutions by digestion with relatively large quantities (3 to 24 per cent) of an ion exchange agent (Decalso), followed by filtration through improved Seitz asbestos pads.

Chemical detoxification of pyrogen has received but limited attention, primarily because of the destructive effect of reagents upon medicinal agents, and because of the difficulties of removing the excess reagent and its by-products. Co-Tui (5) has reported that pyrogen may be destroyed by boiling for one-half hour with 0.1 *N* HCl. Carter (6) has found dilute potassium permanganate solution useful in destroying pyrogen prior to distilling water in all-glass stills. The results of the researches of Robinson and Flusser (7), indicating the polysaccharide nature of pyrogen, suggest the possibility of destroying pyrogen activity by combined hydrolytic cleavage and oxidation.

Of the many available oxidizing agents, hydrogen peroxide would appear to be one of the most promising for depyrogenizing aqueous solutions, since water is its only end product. Obviously it may be used only where, in the concentration employed, it exerts no destructive effect upon the therapeutic or other ingredients of the parenteral solution. There is, however, a dearth of literature on this use of hydrogen peroxide. Hort and Penfold (8) reported in 1912 that centrifuged cells of *Eberthella typhosa*, when washed with hydrogen peroxide, lost their fever-producing effects when injected intravenously. Campbell and Cherkin (9), in a preliminary report of their investigation of plasma substitutes, observed that gelatin solution was rendered nonpyrogenic by boiling with 0.1 *M* H_2O_2 for two hours, or by autoclaving with 0.04 *M* H_2O_2 at 116° for twenty minutes. They did not, however, indicate the effect, if any, of the peroxide upon the gelatin. Nor did they indicate that any excess hydrogen peroxide present after boiling was removed. It should be noted that the liberation of oxygen by catalase when hydrogen peroxide is introduced intravenously may give rise to gas bubbles, if local oxygen solubility is exceeded, with possible fatality from embolism.

It is the purpose of the present study to evaluate hydrogen peroxide as a pyrogen detoxifier in some U. S. P. injections which are intended for intravenous infusion in relatively large volumes.

EXPERIMENTAL

The procedure employed consisted of preparing a concentrated pyrogenic broth and adjusting its potency to a specific number of minimum pyrogenic doses (*M. P. D.*) per 10 cc. injected per Kg. of body weight of rabbit. [Co-Tui (10) has defined the *M. P. D.* as the dose per Kg. of body weight which upon intravenous injection will cause a rise of 0.5 to 0.6° within four hours. Throughout this work the U. S. P. threshold of 0.6° within three hours has been used.] The standardized pyrogenic stock liquid was then added in increments up to 1000 *M. P. D.* per 10 cc. to Water for Injection, Normal Saline Solution, and Dextrose and Sodium Chloride Solution. The pyrogenic solutions were then treated

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with hydrogen peroxide in different concentrations and under varying conditions. The excess peroxide was then decomposed and the solutions filtered aseptically and injected into rabbits to determine their pyrogenic effect.

A Difco nutrient agar slant of *E. typhosa* (Type No. 6539 F. D. A., Hopkins Strain 26) was prepared, and after incubation for forty-eight hours the bacterial growth was washed off with Difco nutrient broth. The bacterial suspension was made up to one liter with broth and incubated at 37° for forty-eight hours. Based on plate count and nephelometric readings, the bacterial population was estimated at 4×10^8 per cc. This stock pyrogen solution was autoclaved at 15 lb. for fifteen minutes and checked for sterility. Dilutions ranging from 20 cc. per 100 cc. to 0.01 cc. per 100 cc. were made in sterile pyrogen-free saline solution, and the solutions filtered aseptically through Selas 015 porcelain filters (1.4 micron porosity). The solutions were injected intravenously in dosage of 10 cc. per Kg. in New Zealand rabbits weighing 1.5 to 2.65 Kg. The 0.01 cc./100 cc. dilution gave a maximum rise of 0.35°. The 0.02 cc./100 cc. dilution gave a maximum rise of 0.62°, representing 1 *M. P. D.* The highest concentration used, 20 cc. of pyrogen stock solution, representing 1000 *M. P. D.*, gave a maximum rise of 2.80°, and resulted in one fatality.

Water for Injection, Normal Saline Solution, and Dextrose and Sodium Chloride Injection (5% dextrose and 0.9% sodium chloride) were prepared and rendered pyrogen free by Ertel filtration, and autoclaved for fifteen minutes at 20 lb. for the water and normal saline, and for sixteen minutes at 11 lb. for the dextrose-saline. They were checked for sterility and freedom from pyrogens. Increments of stock pyrogen solution, corresponding to the amounts of *M. P. D.* indicated in Tables I and II, were added to the injection fluids. Treatment with hydrogen peroxide was carried out in one of three ways:

Method 1.—Using a reflux condenser bearing a sintered glass vent, the solution was boiled for one hour with H_2O_2, in concentrations ranging from 0.02% to 1%. After cooling to 90°, the residual peroxide was decomposed with small portions, 5 × 0.02 Gm., manganese dioxide (N. F. Reagent) per 100 cc. of solution, care being taken to avoid overfoaming during the first few minutes. The liquid was then boiled for five minutes to remove dissolved oxygen, and filtered aseptically through a Selas 02 candle (0.85 micron porosity) into a pyrogen-free container. Intravenous injections of 10 cc. per Kg. of body weight were made within an hour after preparation. A 30% H_2O_2 was used to minimize the volume change due to peroxide addition. Experi-

TABLE I.—DETOXIFICATION OF PYROGEN IN NORMAL SALINE SOLUTION

Expt.	Pyrogen Added before Treatment: Stock Pyrogen, Cc./100 Cc.	Pyrogen Added before Treatment: *M. P. D.*/Kg.	H_2O_2, %	Method of Treatment	MnO_2, Mg./100 Cc.	Maximum Temperature Rise, °C.	Average of Maximum Temperature Rises, °C.
A	1	50	0.5	3	..	1.15	1.01
B	1	50	0.1	1	..	0.35	0.30
C	1	50	0.02	2	0.5	0.58	0.55
D	2	100	1.0	3	..	2.05	1.78
E	2	100	1.0	2	10.0	0.75	0.73
F	2	100	0.1	2	0.5	0.45	0.45
G	5	250	1.0	2	0.5	0.20	0.10
H	5	250	0.5	2	10.0	1.00	0.70
I	5	250	0.2	2	0.5	0.50	0.48
J	5	250	0.1	2	0.5	0.45	0.40
K	5	250	0.02	1	..	1.00	0.72
L	5	250	0.02	2	0.5	0.80	0.72
M	20	1000	1.0	2	10.0	0.80	0.76
N	20	1000	0.1	2	10.0	2.50	2.35
O	20	1000	0.1	2	0.5	0.52	0.50
P	20	1000	0.1	2	0.5	0.30	0.20
Q	20	1000	0.1	1	..	0.44	0.42

TABLE II.—DETOXIFICATION OF PYROGEN IN DEXTROSE AND SODIUM CHLORIDE INJECTION

Expt.	Pyrogen Added before Treatment: Stock Pyrogen Cc./100 Cc.	Pyrogen Added before Treatment: *M. P. D.*/Kg.	H_2O_2, %	Method of Treatment	MnO_2, Mg./100 Cc.	Charcoal, Mg./100 Cc.	Maximum Temperature Rise, °C.	Average of Maximum Temperature Rises, °C.
A	5	250	1.0	3	..	...	1.50	1.47
B	5	250	None	1 hr. boil	5	...	1.30	1.20
C	5	250	0.1	1	..	...	0.55	0.42
D	5	250	0.1	2	5	...	0.24	0.10
E	5	250	0.1	2	..	100	0.24	0.18
F	20	1000	0.1	2	5	...	0.15	0.12
G	20	1000	0.1	2	..	100	0.31	0.20

ments were limited to one hour of boiling after preliminary tests showed that one-half hour was inadequate.

Method 2.—The twofold purpose of this method was to release oxygen continuously in minute quantities for the duration of boiling, and to reduce the amount of peroxide remaining to be decomposed after boiling. Therefore, small quantities of manganese dioxide, as indicated in Tables I and II, were added to the solutions prior to boiling. After one hour of boiling under reflux, any residual peroxide was decomposed by manganese dioxide (0.1 Gm. per 100 cc.), and the balance of the procedure followed as in Method 1.

Method 3.—The solution was allowed to stand for twelve hours with hydrogen peroxide at room temperature (20–25°) under a sintered glass vent. Then 0.1 Gm. manganese dioxide per 100 cc. was added cautiously in 0.02-Gm. increments, and after fifteen minutes, when most of the peroxide had been decomposed, the solution was brought to a boil under a reflux and heated for five to ten minutes to complete the peroxide decomposition and expel dissolved oxygen. The solution was then filtered through a Selas candle as in Method 1.

In all cases, the solutions were tested qualitatively for freedom from peroxide before being injected into the test animals.

In a few instances activated charcoal (Nuchar) was used to replace manganese dioxide as peroxide decomposing agent, for reasons to be explained below.

Table I shows the extent of pyrogen detoxification in normal saline solution. The results for injection water were found to parallel so closely those of the normal saline as to preclude the use of a separate table.

Experiment F, Table I, was repeated, using activated charcoal (0.1 Gm. per 100 cc.) in place of manganese dioxide. Upon injection this produced a maximum rise of 0.4° and an average maximum rise of 0.33°.

Table II shows the extent of detoxification of pyrogen in Dextrose-Saline Solution, manganese dioxide or activated charcoal serving as peroxide decomposing agents.

An examination of the data of both tables makes it readily apparent that hydrogen peroxide used at room temperature (Method 3) is not a satisfactory pyrogen detoxifier up to 1% concentration. Higher concentrations would not prove practical. Additional experiments carried out at room temperature showed that this method was not improved by providing for continuous evolution of oxygen by the addition of 0.5 to 50 mg. quantities of MnO_2 per 100 cc. of peroxide-treated injection fluids. On the other hand, boiling for an hour with peroxide (Method 2) in concentrations as low as 0.02% detoxified moderately large amounts of pyrogen (50 *M. P. D.* per 10 cc.) and 0.1% peroxide under the same conditions detoxified 1000 *M. P. D.* per 10 cc. injection fluid.

Although there are indications that Method 2 is slightly superior to Method 1, the limited number of experiments does not warrant any positive conclusions. However, the small amount of MnO_2 does provide smoother boiling, and reduces or eliminates the residual peroxide to be decomposed. In dealing with large volumes of parenteral solutions, too rapid addition of manganese dioxide at the end of boiling in Method 1 may cause copious frothing for a few minutes. It is desirable, therefore, that the residual peroxide not exceed a few hundredths of a percentum. Method 2 accomplishes this purpose.

On the other hand, too much MnO_2 during the hour of boiling with hydrogen peroxide, even 10 mg. per 100 cc. in the case of saline (Experiments E, H, M, and N, Table I), does not permit effective peroxide detoxifying action. That the manganese dioxide *per se*, in the absence of hydrogen peroxide, is ineffective at boiling temperature as a pyrogen detoxifier, can be seen from the control Experiment B, Table II.

Although MnO_2 proved satisfactory in decomposing residual peroxide in water and saline injections, it produced a slight discoloration in dextrose-saline. Therefore Nuchar was used in its place. Experiments E and G, Table II, show that it is effective, though higher concentrations than for MnO_2 are required. The experiments with MnO_2 were nevertheless carried out, and are included in Table II to permit proper evaluation of hydrogen peroxide as a pyrogen detoxifier and avoid introduction of an extraneous factor, namely, the adsorption of pyrogen by activated charcoal.

When the problem of detoxification of pyrogen by hydrogen peroxide was first approached, it became apparent that the removal of excess peroxide would have to be successfully solved if the method were to prove serviceable. A search of the literature and some preliminary experiments narrowed the field of suitable peroxide deactivating agents to two substances of practical interest, manganese dioxide and activated charcoal. Although both of these may decompose higher concentrations of hydrogen peroxide with explosive violence, the rate of peroxide decomposition at 0.1% H_2O_2 concentration is readily controlled. To determine suitable concentrations of manganese dioxide and charcoal, the experiments listed in Tables III and IV were conducted at the boiling point of water, using saline and dextrose-saline injections, respectively. Residual peroxide concentrations were determined by titration with standard potassium permanganate solution. Dextrose did not interfere in the dilution at which the titrations were carried out.

It is of interest to note that water and saline behave similarly with respect to the influence they exert upon manganese dioxide and activated charcoal as peroxide decomposing agents. The data also provide the reason why 10 mg. of MnO_2 per 100 cc. proved unsatisfactory in Experiments E, H, M, and N in Table I, since effective peroxide concentration does not last more than a few minutes at the boiling point in the presence of this quantity of MnO_2. Dextrose exerts a retarding effect on the decomposition of peroxide by both agents; therefore more is required of either to bring the peroxide level down to the concentration present in water or saline during the boiling process. About 5 to 10 mg. MnO_2 per 100 cc., or 100 to 200 mg. activated charcoal were optimum for the reagents used.

Experiments conducted at room temperature showed that in the absence of manganese dioxide or charcoal, after a twelve-hour digestion about 75% of the original concentration of hydrogen peroxide is retained in the water and saline injections, and over 90% is retained in the dextrose-saline, when an initial concentration of 0.1% H_2O_2. was used.

TABLE III.—RESIDUAL CONCENTRATIONS OF HYDROGEN PEROXIDE IN INJECTION WATER, NORMAL SALINE SOLUTION, AND DEXTROSE-SALINE INJECTION AFTER BOILING WITH MANGANESE DIOXIDE AND 0.1% HYDROGEN PEROXIDE

Time of Boiling, Min.	Percentage of Hydrogen Peroxide								
	Injection Water MnO_2: Mg./100 Cc.			Normal Saline Solution MnO_2: Mg./100 Cc.			Dextrose-Saline Injection MnO_2: Mg./100 Cc.		
	0	0.5	5.0	0	0.5	5.0	0	5.0	5.0
15	0.080	0.043	0.016	0.072	0.050	0.012	0.095	0.092	0.072
30	0.060	0.014	0.000	0.042	0.022	0.000	0.092	0.086	0.064
45	0.027	0.007	0.000	0.020	0.012	0.000	0.089	0.071	0.058
60	0.020	0.003	0.000	0.010	0.003	0.000	0.085	0.068	0.036

TABLE IV.—RESIDUAL CONCENTRATIONS OF HYDROGEN PEROXIDE IN INJECTION WATER, NORMAL SALINE SOLUTION, AND DEXTROSE-SALINE INJECTION AFTER BOILING WITH ACTIVATED CHARCOAL AND 0.1% HYDROGEN PEROXIDE

Time of Boiling, Min.	Percentage of Hydrogen Peroxide								
	Injection Water Charcoal: Mg./100 Cc.			Normal Saline Solution Charcoal: Mg./100 Cc.			Dextrose-Saline Solution Charcoal: Mg./100 Cc.		
	0	100	1000	0	100	1000	0	100	1000
15	0.080	0.056	0.006	0.072	0.046	0.005	0.095	0.066	0.017
30	0.062	0.044	0.000	0.042	0.030	0.000	0.092	0.058	0.000
45	0.027	0.022	0.000	0.020	0.014	0.000	0.089	0.048	0.000
60	0.020	0.017	0.000	0.010	0.008	0.000	0.085	0.040	0.000

It should be noted that the data in Tables III and IV apply to the particular deactivating reagents used in this study. Particle size and other variables may influence the rate of peroxide decomposition. Several grades of manganese dioxide tested did not produce significantly different results from those in the tables. However, activated charcoals differ considerably in properties, and preliminary tests of their rates of decomposing H_2O_2 should be made before applying them for this purpose.

All three injection fluids were examined after processing by Method 2. The *p*H of the water was 5.7 before treatment; it was 6.2 after treatment when charcoal was used, and 6.9 when MnO_2 was used. The rise appears due to CO_2 expulsion during boiling. The water after treatment met the U. S. P. permanganate test for limit of oxidizable substances. The total solids after treatment was 0.7 mg. per 100 cc. (U. S. P. limit is 1.0 mg.). It should be noted that since the U. S. P. permissible limit of acid-soluble substances in activated charcoal is 35 mg. per Gm., charcoal should be boiled with dilute hydrochloric acid and washed with water prior to use.

The *p*H of the saline solution changed only slightly, from 6.8 to 6.68 when manganese dioxide was used, and to 6.9 after charcoal treatment. The sodium chloride content was unaltered. In both saline solution and water, no traces of hydrogen peroxide or manganese ion were detected. The liquids were odorless and colorless.

Examination of the dextrose-saline after treatment with peroxide and manganese dioxide showed that the total acidity remained within U. S. P. limits for dextrose. The *p*H dropped from 6.86 to 5.9. When charcoal was used it dropped to *p*H 5.2. It is usual for the *p*H of dextrose solutions to drop after heat sterilization. A slight yellow color and a trace (0.5 p. p. m.) of manganese ion were found in the dextrose-saline after filtration. Therefore, manganese dioxide should be replaced by activated charcoal in the treatment of dextrose solutions. The charcoal yields a colorless filtrate.

The possibility of dextrose oxidation by the peroxide was next examined. Payne and Foster (11) have shown that when dextrose is treated with hydrogen peroxide, using 2 to 5 moles of peroxide per mole of dextrose, and heating for eighteen to twenty-four hours, about 50% to 80% is converted to gases, mainly CO_2, and about 1% to 40% is oxidized to aliphatic acids. However, such rigorous oxidation cannot be compared with an hour of boiling at a concentration of 0.1% H_2O_2. Polarimetric examination of the dextrose-saline solution after peroxide treatment showed only a slight drop in optical rotation, from 4.84° to 4.76° after the use of manganese dioxide, and from 4.84° to 4.81° when activated charcoal was used as peroxide decomposing agent. It is probable that a slight amount of gluconic acid or other carboxylic acid was formed which accounts for the slight drop in *p*H. However, it is known that a similar slight oxidation occurs even in the absence of H_2O_2, when dextrose solution is autoclaved in commercial practice. The trace of manganese ion found in the dextrose saline solution could be accounted for on the basis of the solvent action of the carboxylic acids on the manganese dioxide. By replacing the MnO_2 with activated charcoal, this problem is circumvented.

DISCUSSION

The peroxide method of detoxifying pyrogenic solutions is worthy of consideration where no oxidative changes are produced in the active ingredients, and where the requisite H_2O_2 concentration may be maintained during boiling. Peroxide concentrations of the order of 0.05% to 0.1% are desirable during the first fifteen minutes of boiling. Concentrations of 0.003% to 0.03% are desirable after an hour of boiling. It may also be noted that for water and saline, but not for dextrose solutions, boiling for two hours with an initial concentration of 0.1% H_2O_2 has been found in most instances to yield a peroxide-free solution, obviating the need of a peroxide decomposing agent.

The peroxide method, where applicable, offers

such advantages over adsorption filtration as freedom from asbestos fibers or haze in the filtrate, simplicity of cleaning and sterilizing the filter unit, capability of detoxifying greater amounts of pyrogen, and applicability to other than crystalloidal solutions.

SUMMARY

A method for detoxifying pyrogens in water, saline, and dextrose-saline solutions has been tested, using a calibrated pyrogen preparation derived from *E. typhosa*.

It has been found that boiling with 0.1% of hydrogen peroxide for one hour is capable of detoxifying 1000 minimum pyrogenic doses per 10 cc. in these injection fluids.

For the removal of residual peroxide, a limited quantity of manganese dioxide, during the boiling, is suggested for use in water or saline; purified activated charcoal, acid- and water-washed, should be used for this purpose for dextrose solutions. Filtration through an 02 Selas candle removes the peroxide decomposing agent and yields a particle-free solution.

No evidence has been found of significant change in the injection fluids subjected to this method of detoxifying pyrogen.

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The Applications of Radioactive Tracer Techniques to Pharmacy and Pharmaceutical Research*

By JOHN E. CHRISTIAN

A brief review of some of the applications of radioactive isotopes to pharmaceutical research is presented. Subsequent reports will describe experimental procedures and include data on the application of radioactive isotopes to research.

THE PROGRESS of science is characterized by the discovery and use of relatively few instruments or tools for application in research. Examples of such instruments are the analytical balance and the microscope which, when discovered, advanced science rapidly. Within the last fifteen years, however, two very useful instruments have been developed, the "cyclotron" and the "chain reacting pile." The cyclotron is capable of producing high-speed particles of matter of various descriptions and the pile of producing slow neutrons. The products of these instruments, radioactive isotopes, are new tools in the hands of the scientists. It has been stated that the range of usefulness of the products of the cyclotron and the pile is unsurpassed by any other tools at our disposal with the possible exception of the analytical balance and the microscope.

Pharmacy, as a profession, is chiefly the application of all branches of science to the production of medicinals and medicinal products, and since radioactive isotopes are applicable to all branches of science, isotopes have particular application in the various divisions of the pharmaceutical profession. In view of these facts, the pharmaceutical profession should be quick to awaken to the unique opportunities afforded by radioactive isotope "tracer" techniques.

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Isotopes have many important uses in the various fields of science such as development of power, atomic research, etc.; however, the pharmaceutical profession is primarily concerned with the use of products which are radioactive.

APPLICATIONS

Radioactive products have at the present time two very outstanding applications to pharmacy and pharmaceutical research: (1) as therapeutic agents when used properly and with necessary precautions and (2) as tracer elements. As therapeutic agents, radioactive isotopes by virtue of their radiations are, in the true sense of the word, a drug, and are so classified and regulated under Section 505 of the Federal Food, Drug, and Cosmetic Act.

The particles emitted when the isotope disintegrates are a source of penetrating radiation in the treatment of abnormal tissues, a circumstance in which the injury to unwanted tissue is notably greater than to normal essential tissue.

Radioactive Isotopes in Pharmaceutical Research.—In pharmaceutical research the use of radioactive isotopes in therapy offers several possibilities. Specific localizing compounds, which, by preferentially localizing in diseased tissue, will result in much greater radiation damage to the diseased than to normal tissue, need to be prepared and tested. A few such studies have been made, examples of which are iodine and certain gold colloids, but a great variety of compounds, and information concerning their concentration in various tissues, should be investigated. Perhaps much of this information may be forthcoming from data obtained from other studies.

A second possible application of radioactive isotopes concerning therapeutic agents is the production of modified forms of materials commonly prepared by manufacturing pharmacists. The effects of radiation in the production of modified strains of bacteria, viruses, fungi, and actinomyces, and such effects as the denaturation of proteins and inactivation of enzymes should be studied.

A third and very important study is that of the nature and method of treatment of radiation damage. In this direction drugs and necessary treatment should be developed to prevent or lessen radiation damage and sickness. This study is important for cases of possible human over exposure in radiation therapy, atomic energy work, and warfare.

Radioactive isotopes have already been used as diagnostic agents; however, much remains to be done toward extending this usage.

In regard to therapeutic usage of isotopes there is little direct commercial application in which the pharmacist, or manufacturing pharmacist will be interested since there is no immediate prospect of the use of isotopes in medicine in large amounts. The conditions in which isotopes have particular value therapeutically are rare at the present time and in such cases the dose is small and varied. The discovery of specific localizing agents as mentioned above might change this picture appreciably.

Application as Tracer "Elements".—Radioactive isotopes, as tracer "elements" rather than as therapeutic agents, have the principal applications to pharmacy and pharmaceutical research at the present time. A radioactive isotope of an element behaves identically with other isotopes of the element in all chemical and physiological processes. It labels without question the particular atoms one wishes to trace regardless of their incorporation into units with other atoms or into any combination in which one might desire to trace them. In other words, we have the ultimate in specificity for the tagging of an atom or element.

The advantages of the use of radioactive isotopes as tracer substances may be listed:

(1) The movement of tagged substances is easily followed using suitable detection devices.

(2) Small amounts of the tagged substance may be used. As an example, as little as 10^{-7} Gm. of iodine, 0.1 microgram, is sufficient for the tracer technique using radioactive iodine.

(3) It is often unnecessary to remove organs or tissues or to kill the organism in order to determine the presence and content of radioactive material.

(4) The detection of elements by their radioactivity is up to one million times as sensitive as by the usual methods of chemical or physical analysis. This means that roughly 10^{-18} to 10^{-20} Gm. of substance can often be readily detected.

Such sensitivity of measurement makes possible the intelligent study of problems which heretofore could not be solved by the use of existing methods or tools. In pharmacy and in pharmaceutical research in particular, we have many problems which lend themselves to the application of radioactive isotopes and which cannot be adequately solved by other existing methods.

Pure Pharmacy Research.—As a tool in pure pharmacy research, radioactive isotopes have widespread application. A few of the possible applications may be briefly mentioned here as illustrations of this point.

Researchers have long desired to know the rate of absorption of certain medicaments from various bases so that bases could be classified in this respect. Also it is desirable to know if certain medicaments pass through the normal skin and membranes at all and if so to what extent. Since such small amounts of materials are involved, isotopes offer about the only method of measuring such absorption. The rate of absorption of certain substances varies with the degree of irritation of the skin, thus the need for a method of measuring skin irritation is apparent.

The disintegration rate of tablets and enteric-coated pills and tablets *in vivo* may be most critically studied using radioactive isotopes. The problem of exactly when and where an enteric coat breaks down in the animal body is of considerable importance in determining the relative efficiencies of the various enteric coats.

The pharmaceutical profession has a variety of methods for the administration of a specific medicinal substance. The question of just which type of preparation gives the most satisfactory absorption or utilization of the medicinal can now be determined precisely by means of the tracer technique. These are but a few of the many examples which might be cited for the use of isotopes in pure pharmacy research.

Tracer Technique in Pharmacology.—In pharmacology as applied to pharmacy, the tracer tech-

nique offers even greater possibilities for study.

Wherever one desires to measure small amounts of substances this technique is particularly applicable. The determination of small amounts of residues on the skin and of the effect of various solutions upon the calcium-phosphorus depletion of teeth, both of which are to be reported on in subsequent papers, are excellent examples of tracer application. Studies such as these are next to impossible without the isotope tracer technique.

One of the greatest advantages which isotopes offer in pharmacology is making possible a study, not previously feasible, of the rate of absorption, excretion, alteration, breakdown, concentration in the blood, and per cent distribution in various organs of almost every synthetic medicinal substance. The same type of study may also soon be done with certain natural substances which are not readily synthesized, since a plant farm designed to grow plants in an atmosphere of radioactive CO_2 is now being set up in Chicago. This unit is being designed to produce natural drug principles containing radioactive atoms. To trace in the body such substances as vitamins, hormones, antitoxins, drugs, etc., which distribute themselves in tissue in minute quantities is impossible by ordinary analytical procedures. The value of such studies cannot be predicted; however, one can say that undoubtedly the contribution will be much greater than can be anticipated. Even the study of such a simple substance as ethyl alcohol would be a valuable contribution to pharmacy and medicine.

By use of the radioautographic technique more detailed tracer studies can be made, determining exactly in what cells within a particular organ the substance being studied concentrates.

Radioactive isotopes also lend themselves to toxicity studies, a major area of pharmacological study.

The study of the uptake, utilization, storage, mechanism of action, and need in the animal body of certain trace elements such as Cu, Co, Ni, Cr, etc. is now possible by the tracer technique. In short, we may say that radioactive isotopes are applicable to almost every pharmacological problem.

Practical Applications in Pharmaceutical Chemistry—In the field of pharmaceutical chemistry, the applications of radioactive isotopes are concerned primarily with the synthesis of substances which contain radioactive atoms which may then be traced. Such syntheses require special techniques and precautions, but are not considered difficult or dangerous.

The applications of isotopes in pharmaceutical chemistry, however, are not confined to synthesis. Radioactive isotopes offer the most sensitive quantitative method known and, as such, provide a number of practical applications. The determination of the solubilities of very slightly soluble compounds is not possible to the same degree by other existing methods. The use of isotopes for such determinations possesses the advantage that the presence of foreign ions in no way interferes with the measurements. The method may be applied without difficulty, for example, in determining the solubility of lead sulfate in the presence of calcium sulfate. The determination of the amount of a given substance that is absorbed or occluded during a precipitation or purification procedure offers considerable application. The determination of mere traces by ordinary chemical analysis, when it can be accomplished at all, requires tedious methods and is time consuming. Isotopes also lend themselves to a study of the behavior, chemical reactions, and properties of medicinal substances of unknown properties.

Colloids are extremely important in pharmaceutical and medicinal chemistry and radioactive isotopes are ideal for their study from almost every standpoint. Such studies would reveal considerable information about colloids which is not used or known today.

In conjunction with co-precipitation, elution, added carriers, and chromatographic absorption procedures, it will be possible to isolate, purify, and identify substances in small amounts from complex mixtures which heretofore was not possible. Such procedures used along with pharmacological studies will be invaluable to pharmaceutical chemical research.

Research Area in Pharmacognosy.—In the field of pharmacognosy, there are likewise many opportunities for applications of radioactive isotopes. Here again a few examples will suffice to illustrate the use.

Of prime importance at the present time is the production of natural plant products which are radioactive. With many alkaloids, glycosides, etc., this is only possible by the growth of plants from which these materials are obtained in an atmosphere of radioactive carbon dioxide or in a media containing the desirable tagging atom. The problems of production and isolation of such principles fall within the scope of the pharmacognosist. The use of radioactive isotopes in conjunction with the field of hydroponics offers a most fertile field for the production and study of natural plant medicinals. A study by the radioautographic technique of just what plant cells contain the active principles and at what periods the content is greatest in the various cells would be invaluable. The pharmacognosist can contribute to the production of radioactive antibiotics which in itself is an important research area. The rate of uptake, distribution, and elimination of various elements, radicals, and compounds in medicinal plants is a research project worthy of considerable study, especially in conjunction with the effect of various substances upon the active principles produced by the plant.

A study of the need of medicinal plants for certain trace elements and fertilizer needs likewise can now be investigated more thoroughly using the tracer technique.

Radioactive isotopes may also be applied to solve certain manufacturing problems, examples of which are the uniform distribution of drugs in various medicinal forms, mixing problems, and various quantitative problems.

SUMMARY

Briefly, we may say that radioactive isotopes are applicable to all branches of pharmaceutical research and by virtue of the very nature of pharmaceutical problems themselves are especially important.

It is hoped that the illustrations of the applications of radioactive tracers to pharmacy and

pharmaceutical research, as presented here, are of sufficient number and variety to show that this tool is capable of many diverse applications.

It is desirable that in the future a greater number of individuals interested in pharmaceutical research and the welfare of pharmacy make use of this excellent research tool and thereby facilitate the solution of many important pharmaceutical problems.

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The Use of a Microcrystalline Wax in Tablet Polishing. I.*

By W. CARL KELLEY†

Microcrystalline waxes have a multiplicity of uses. The production of a polish is very desirable on coated tablets. Two microcrystalline waxes from petroleum are produced by the Bareco Oil Company of Barnsdall, Okla. One melts at 170° F. and the other at 190° F. This paper deals with the wax of lower melting point and evidence is presented to show that when it is mixed with carnauba (or possibly some similar product) a satisfactory polish is produced.

THE POLISHING of coated tablets is an accepted practice in pharmaceutical manufacturing today. Polishing facilitates handling, makes the tablets more pleasing in appearance, and enhances the value of coating. Very little was found in the literature, however, pertaining to the processes or ingredients employed. A carnauba-beeswax mixture in a suitable solvent such as acetone was given by Bennett (1). Glyceryl tristearate was also mentioned as a polishing agent by Bennett. Stokes (2) recommended the application of a solution of carnauba wax in acetone, followed by a small amount of talc upon the evaporation of the solvent. A chloroform solution of carnauba wax and white wax has been suggested by Rose, in which about 100 grains of the waxes was used for fifty thousand five-grain tablets (3). A formula consisting of three parts of carnauba wax, two parts of beeswax, and two parts of paraffin was found in the textbook by Scoville and Powers (4).

Microcrystalline waxes from petroleum are produced in this country. They have been shown to be of value in the preparation of certain pharmaceutical preparations (5). It is the purpose of this paper to report on a preliminary study of the use of a microcrystalline wax for polishing tablets.

EXPERIMENTAL

A small improvised polishing drum, 10 inches in diameter and 8 inches deep, was lined on the inside with a light-weight canvas. It was rotated at the rate of twenty-two r.p.m., by an electric motor.

The microcrystalline wax used was "Bareco 170," which was furnished by the Bareco Oil Company, Barnsdall, Okla. (The 170 refers to the melting point of 170° F.) The Warren-Teed Pharmacal Company of Columbus, Ohio, supplied the brown-coated, unpolished tablets.

Bareco wax was melted, applied to the canvas inside the drum, and allowed to harden at 20°. Five hundred tablets were placed in the drum and the drum rotated. After a time, some modifications were made. Samples of twenty-five tablets each were taken at intervals and labeled as follows:

1. Polished for five minutes.
2. Polished for fifteen minutes.
3. Polished for twenty-five minutes
4. Polished for forty minutes.
5. Polished for one hour, added small amounts of powdered stearic acid and talc and allowed to rotate for an additional five minutes.
6. Same as (5) with an additional thirty minutes' rotation.

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7. Same as (6) but the tablets were placed in the drum lined with cheesecloth and rotated for forty-five minutes in an attempt to remove excess wax and improve the gloss.

The observations made during these tests showed that the tablets began to adhere to the waxed canvas and to each other after about twenty-five minutes of rotation. The wax did not produce a satisfactory gloss. Neither addition of the stearic acid and talc nor buffing with cheesecloth produced satisfactory results.

The formula was modified by melting Bareco wax and carnauba wax in equal proportion to replace the pure Bareco wax. Five hundred tablets were used as before. A good polish was produced after rotating them for approximately three hours.

The formula was again modified by using three parts of Bareco wax to one part of carnauba wax. The same procedure was followed as before. Rotating for from two to two and one-half hours gave very satisfactory results. The tablets had a polish that was comparable to the gloss found on tablets that are on the market.

A test was made to determine the possible effects upon the rate of disintegration when taken into the stomach. Distilled water was warmed to 37°. When the tablets were dropped into it, the color of the coating appeared in the water almost instantly

SUMMARY AND CONCLUSION

1. An excessive amount of wax was deposited on the tablets when the microcrystalline wax (Bareco wax 170) alone was used. This wax coating was dull in appearance and was also "tacky" or "sticky." By the method employed, the tablets used could not be polished satisfactorily by the microcrystalline wax only. Buffing the waxed tablet on cheesecloth does not greatly improve the gloss of the tablet and seems to be impractical.

2. Fifty per cent each of microcrystalline wax and carnauba wax was used to polish the tablet and the resulting gloss was satisfactory. The time required to polish the tablets seemed to be excessive.

3. A formula, consisting of 75 per cent microcrystalline wax and 25 per cent carnauba wax, produced a satisfactory polish on the tablets used. The polish did not interfere with disintegration of the tablets.

It is concluded, from this investigation, that Bareco 170° microcrystalline wax, in combination with carnauba wax, will produce a satisfactory polish on coated tablets.

REFERENCES

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Book Reviews

Jubilee Volume Dedicated to Emil Christoph Barell. By the Scientific Workers of the Roche Companies. Basle, 1946. 468 pp. 15 x 23 cm.

This book is a typical European Jubilee volume dedicated, in this instance, to the president of F. Hoffmann-LaRoche and Company on the occasion of the 50th anniversary of his association with the firm. It represents reviews of scientific work done by members of the staff. Chemistry, pharmacology, mycology, bacteriology, and other scientific fields are represented and such variable compounds as vitamins, intestinal antiseptics, analgesics, adenine, antibiotics, and dicumarol are found in these papers. Eight of the papers are in German.

Inorganic Pharmaceutical Chemistry. By CHARLES H. ROGERS, TAITO O. SOINE, and CHARLES O. WILSON. Fourth edition. Lea and Febiger, Philadelphia, 1948. 704 pp. 23 illustrations. 15.5 x 24 cm. Price, $10.

The first edition of this well-known pharmaceutical text and reference book was published in 1930. Since that time it has undergone three revisions. The fact that it is now in its fourth edition should be a sufficient indication of its utility and value to pharmacists, teachers of pharmacy, and students in pharmacy. The fourth edition of this widely used textbook has been thoroughly revised and brought up to date. It not only reflects the most recent advances in inorganic pharmaceutical chemistry but conforms to the current editions of the United States Pharmacopœia and the National Formulary.

A significant feature of the new edition is the more detailed discussion of therapeutic value and medicinal uses of inorganic compounds and their preparations. The style and arrangement of the chapters are essentially the same as in previous editions. The book should, as it has in the past, serve a particularly useful purpose as a textbook for the correlation of general and inorganic chemistry with inorganic medicinal chemicals. The binding and printing are adequate and the book is well indexed. Those familiar with previous editions will not be disappointed when they examine and use the new fourth edition.